UNIVERSITY OF TASMANIA

NOVEL *CHLAMYDIA*-LIKE AGENTS OF EPITHELIOCYSTIS IN WILD AND CULTURED AUSTRALIAN FINFISH

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This thesis is dedicated to David McLennan who showed me how to explore life's curiosities, and to Mayja Cailing who told me never to lose my innocence.
Both inspired me to follow the scientists' dream.

APPROVALS

Doctor of Philosophy Dissertation

Epitheliocystis in Australian Finfish

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STATEMENT OF ORIGINALITY

I declare that this is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been duly acknowledged in the text and a list of references if given.

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STATEMENT OF ETHICAL CONDUCT

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

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LIST OF ABBREVIATIONS

The following abbreviations are used in this thesis:

+ve	positive
-ve	negative
α-DIG-AP	alkaline phosphatase-labeled anti-DIG Fab fragments
ANOVA	analysis of variance
BCIP/NBT	5-bromo-4chloro-3-indolyl-β-D-galactopyranoside/Nitro Blue Tetrazolium
BLAST	basic local alignment search tool
bp	base pairs
C	cultured
Ca.	*Candidatus*
Cap.	captive
CIPRES	Cyberinfrastructure for Phylogenetic Research
CLO	*Chlamydia*-like organism
DIG	digoxigenin
df	degrees of freedom
DNA	deoxyribonucleic acid
EB	elementary body
EDTA	Ethylenediaminetetraacetic acid
ELO	epitheliocystis-like organism
EM	electron microscopy
F	F statistic
FAO	Food and agriculture organisation
fem.	feminine
FISH	fluorescent *in situ* hybridisation
FW	Freshwater
g	grams
gen.	genus
GPS	Global positioning system
h	hour
H&E	Haematoxylin and Eosin

HCl	hydrochloric acid
HSD	honestly significant difference
IB	intermediary body
ICC	immunocytochemistry
IHBI	Institute of Health and Biomedical Innovation
IHC	immunohistochemistry
ISH	*in situ* hybridisation
kg	kilogram
KW	Kruskal-Wallis
L	litre
M	marine
mg	milligram
min	minute
mL	millilitre
mm	millimetre
mM	micromole
n	number of samples
NAPS	nucleic acid preservation solution
NCBI	National Centre for Biotechnology Information
NCMCRS	National Centre for Marine Conservation and Resource Sustainability
nd	no data
N.L.	novel lineage
nm	nanometre
nov.	novel
p	statistical p-value
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEN	polyethylene naphthalate
pmol	picomole
RB	reticulate body
RLO	*Rickettsia*-like organism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid

SE	standard error
sing.	single
sp.	species
SSC	saline-sodium citrate
SNP	single nucleotide polymorphism
ST	striped trumpeter
SW	southwest
TAFI	Tasmanian Aquaculture and Fisheries Institute
TE	Tris and EDTA buffer
TEM	transmission electron microscopy
UPGMA	unweighted-pair group method using average linkages
W	wild
YTK	yellowtail kingfish
µm	micrometre
µL	microlitre

LIST OF COMPUTER PROGRAMS USED FOR PHYLOGENETIC ANALYSIS

CIPRES The CIPRES Science Gateway Version 3.1 is a public resource for inference of large phylogenetic trees. It is designed to provide all researchers with access to large computational resources of the NSF TeraGrid through a simple browser interface. The CIPRES portal is found at http://www.phylo.org/sub_sections/portal/

jModelTest jModelTest is a tool to carry out statistical selection of best-fit models of nucleotide substitution. It implements five different model selection strategies: hierarchical and dynamical likelihood ratio tests (hLRT and dLRT), Akaike and Bayesian information criteria (AIC and BIC), and a decision theory method (DT). Open source software Version 0.1.1 available from http://darwin.uvigo.es/software/jmodeltest.html

MEGA5 MEGA is an integrated tool for conducting sequence alignment, inferring phylogenetic trees, mining web-based databases, estimating rates of molecular evolution, inferring ancestral sequences, and testing evolutionary hypotheses. Open source software Version 5.2.2 available from http://www.megasoftware.net/

MESQUITE Mesquite is software for evolutionary biology, designed to help biologists analyse comparative data about organisms. Open source software Version 2.73 available from http://mesquiteproject.org/mesquite/mesquite.html

MrBayes MrBayes is a program for Bayesian inference and model choice across a wide range of phylogenetic and evolutionary models. MrBayes uses Markov chain Monte Carlo (MCMC) methods to estimate the posterior distribution of model parameters. Open source software Version 3.2.1 available from http://mrbayes.sourceforge.net/

MUSCLE MUSCLE is a program for creating multiple alignments of amino acid or nucleotide sequences. Version 3.7 is available from http://www.drive5.com/muscle/

EXECUTIVE SUMMARY

Epitheliocystis is a *Chlamydia*-like, obligate intracellular bacterial condition that affects the gills and skin of finfish. To date, epitheliocystis have occurred in both wild and farmed fish populations and have been described in over 90 fish species worldwide, including hosts from marine and freshwater environments. In Australia, the condition has been reported from economically important species, such as yellowtail kingfish (*Seriola lalandi*), barramundi (*Lates calcarifer*) and silver perch (*Bidyanus bidyanus*). It is characterised by the presence of membrane-enclosed basophilic inclusions in host epithelial cells and can cause a severe proliferative host response, including swollen gills, excessive mucus production and respiratory distress. Affected fish, especially those in commercial culture, exhibit lethargy, surface gaping and reduced growth. Epitheliocystis can cause mortalities of up to 100% in commercially cultured fish.

While over ten species from Australian waters have been reported with epitheliocystis infections, there is however very little information available on the infection levels of this condition in both wild and farmed fish in Australia. In addition, the diversity of *Chlamydia*-like epitheliocystis agent(s) is not fully known and as it stands, no epitheliocystis agent has been characterised in the Southern Hemisphere.

The overall objective of this thesis was to study the disease epitheliocystis in wild and farmed Australian finfish and to gain a greater understanding of the aetiological agent(s) involved. To address this objective the following specific aims were studied:

a) Investigate and document infection levels in wild and farmed fish, and determine which factors affect epitheliocystis outbreaks
b) Characterise epitheliocystis agents from at least two species of Australian fish
c) Critically review the current understanding of the role of *Chlamydia*-like organisms found in association with epitheliocystis

Much still needs to be learnt about the epidemiology of chlamydial infections in fish. Due to the inherent nature of intracellular bacteria requiring a host cell to survive, there is currently no *in vitro* method to culture these bacteria in pure form. As a result, Koch's

postulates are not possible to fulfil at this time and the molecular postulates developed by Fredricks and Relman were used instead to prove the disease causation relationship.

This thesis presents the results for epitheliocystis in six species of finfish of Australian origin. Three wild caught species, jack mackerel (*Trachurus declivis*), sand flathead (*Platycephalus bassensis*) and tiger flathead (*Neoplatycephalus richardsoni*), were surveyed to document the natural prevalence rates of epitheliocystis in Eastern Tasmanian waters. Histology of gill tissue revealed epitheliocystis to be present in all three species. In addition, there was a significant correlation of increasing prevalence of epitheliocystis with a decrease in seawater temperature. This was the first published report of epitheliocystis in both sand flathead and tiger flathead.

Three commercially farmed species, yellowtail kingfish, striped trumpeter (*Latris lineata*) and barramundi were also studied. Gill tissue from yellowtail kingfish, striped trumpeter and barramundi were sampled for histology, 16S rRNA amplification and sequencing, *in situ* hybridisation and transmission electron microscopy. Epitheliocystis was present in all commercial species tested by histology. DNA analysis revealed the presence of novel *Chlamydia*-like 16S rRNA sequences from gill DNA of all three fish species, and were found to be only 87-88% similar to their nearest known relative, the previously published '*Candidatus* Piscichlamydia salmonis' (AY462244) from Atlantic salmon (*Salmo salar*) and Arctic charr (*Salvelinus alpinus*). Supporting the *Chlamydia*-like result was the *in situ* hybridisation and transmission electron microscopy, revealing that the bacteria inside the cysts were in fact *Chlamydia*-like. From comprehensive phylogenetic analysis, it was found that a novel family lineage, two novel genera lineages and three novel species lineages within the Order *Chlamydiales* had been discovered and these were then described. These novel bacteria were named '*Ca.* Parilichlamydia carangidicola', '*Ca.* Similichlamydia latridicola' and '*Ca.* Similichlamydia laticola', from yellowtail kingfish, striped trumpeter and barramundi, respectively. The most exciting find, a horizontal transmission route of the *Chlamydia*-like bacteria causing epitheliocystis infections in barramundi, is a world first. The molecular characterisation and description of these epitheliocystis aetiological agents reported in this thesis are the first of their kind of *Chlamydia*-like bacteria associated with epitheliocystis from any fish species found in the southern hemisphere.

These chlamydial infections of fish are becoming more broadly recognised by researchers and farmers as an important cause of disease, especially within commercial aquaculture. Aided by advances in molecular detection and typing, recent years have seen an explosion in the description of these epitheliocystis-related chlamydial pathogens of fish, significantly broadening our knowledge of the genetic diversity of the Order *Chlamydiales*. Remarkably, in most cases, each new piscine host studied has revealed the presence of a phylogenetically unique and novel chlamydial pathogen. The work presented in this thesis has made a significant contribution to the knowledge of epitheliocystis and its many aetiological agents.

CHAPTER 1 GENERAL INTRODUCTION

1.1 GLOBAL AND AUSTRALIAN SEAFOOD PRODUCTION

Seafood production is defined to include fish, crustaceans, molluscs and other aquatic animals from both wild caught and aquaculture sources (1). Total global seafood production reached 148.5 million tonnes in 2010, with aquaculture contributing 39.4% to the total production at a value of USD $119.4 billion (1).

Global aquaculture production has increased exponentially in the past 60 years from 639,000 tonne in 1950 to over 78,800,000 tonne in 2010 (1). Aquaculture in Australia has followed a similar trend and is the country's fastest growing primary industry, with total production of 75,188 tonne valued at $948.1 million in 2010/11 (2). There are over 40 species being commercially produced in Australian aquaculture, with 85% of production coming from five high value species; southern bluefin tuna (*Thunnus maccoyii*), pearl oysters (*Pinctada maxima*), Atlantic salmon (*Salmo salar*), edible oysters (*Crassostrea gigas* and *Saccostrea glomerata*) and prawns (*Penaeus monodon* and *Fenneropenaeus merguiensis*) (2). While these established species contribute significantly to Australia's aquaculture industry, new species are continually being assessed for commercial production. In the last decade along, production has increased substantially through improved knowledge of culturing techniques, animal husbandry methods, nutritional requirements and a greater understanding of diseases and their pathways.

1.1.1 Yellowtail kingfish production

Yellowtail kingfish (*Seriola lalandi*) is relatively new to commercial aquaculture production in Australia and New Zealand (3). Yellowtail kingfish are produced in several locations across Australia, however the largest commercial producer of this fish species in the Southern Hemisphere is Clean Seas Tuna Limited (Clean Seas), based out of Port Lincoln, South Australia (Figure 1.1). The company produce fingerlings in house, which are transferred to growout cages where they are grown to a marketable size of 1.5-3kg. While Clean Seas only produce *S. lalandi*, other *Seriola* species that have been commercially produced around the world include *S. mazatlana* (4), *S. dumerili* (5) and *S.*

quinqueradiata (6). Currently, total world aquaculture production of *Seriola* spp. has increased from approximately 1 tonne in 1999 to 20 tonne ten years later (1).

Figure 1.1: (A) YTK cage aquaculture in Port Lincoln, South Australia (Photo credit: Prof. Ken Cain, University of Idaho); (B) YTK after harvest in Port Lincoln, South Australia.

Yellowtail kingfish are serial spawners that breed in summer and/or autumn (3), depending on seasonal sea temperature ranges of particular locations. However, a major setback in the production of any *Seriola* species has been its susceptibility to diseases such as the monogenean parasites *Zeuxapta seriolae* and *Benedenia seriolae* (7-14), lymphocystis, plasteurellosis (15) and epitheliocystis (5, 16, 17). Many of these authors have reported mortalities due to a proliferative host response, however with a record of co-infections, a determination of an infection reaction pattern and a definitive cause of mortality is often difficult.

1.1.2 Striped trumpeter production

The striped trumpeter (*Latris lineata*) is a deep-water pelagic species that is found in temperature waters of the Southern hemisphere across the Atlantic, Indian and Pacific Oceans (18). The species is an opportunistic carnivore found over rocky reefs at depths from 50 to 400 m. It is also long-lived (~43 years), growing up to 120cm (18). The species is a highly prized eating and game fish, however it has been overexploited throughout its natural range, leading to a significant drop in the wild population. As a result, the total commercial catch has decreased to just 12.8 tonne in 2009/10 (19, 20).

This high value species has been in development for commercial aquaculture production in Tasmania, Australia, since the early 1980s (Figure 1.2) (21). It was selected as a candidate for aquaculture due to their docile nature, lack of cannibalism, ability to take formulated feeds and their tolerance to being held in captivity at high densities (19). Early research into the larval development however, was considerably harder than first thought due to the extended and complex life cycle of the species. As a result, the diverse research program at the Tasmanian Aquaculture and Fisheries Institute, Hobart, focused into four key areas; 1) control of reproduction, 2) early larval development, 3) larval nutrition and 4) larval health (19). As a result of this concerted research effort, there is now fully functional methods and protocols for the commercial production of striped trumpeter (21). However due to economic reasons, there is currently no commercial aquaculture production of this species.

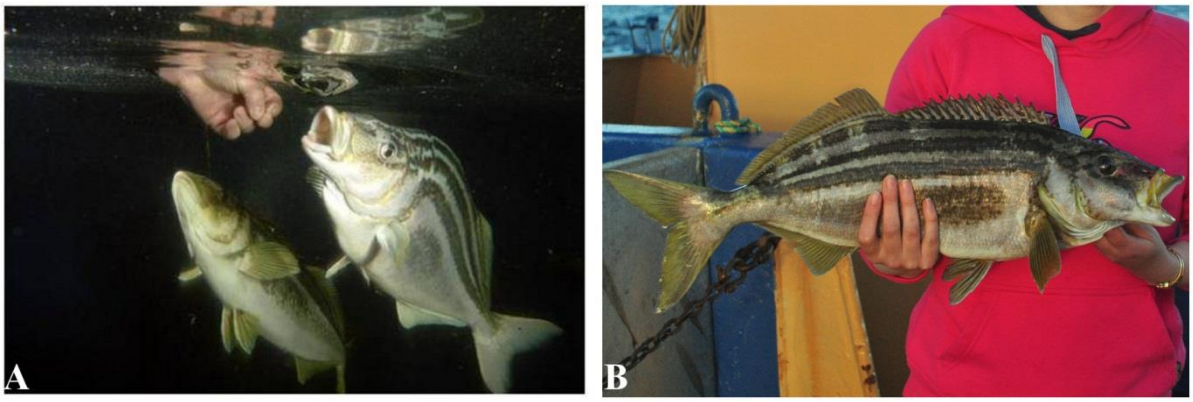

Figure 1.2: (A) Striped trumpeter, *Latris lineata*, that were bred in captivity at TAFI, Hobart (Photo credit: TAFI, Hobart); (B) Wild striped trumpeter caught by fisherman in East Tasmanian waters.

1.1.3 Barramundi production

The barramundi, *Lates calcarifer* (Bloch), is a euryhaline species that spends time throughout its lifecycle in marine, estuarine and freshwater environments. The species is naturally distributed throughout northern Australia, from Ashburton River (22°30'S) on the west coast, to Noose River (26°30'S) on the east coast (22).

Figure 1.3: (A) Barramundi, *Lates calcarifer*; tank based cultured; (B) mature broodstock; (C) barramundi fingerlings (Photo credit: www.robarra.com.au)

Barramundi aquaculture in Australia began in 1983 at the Northern Fisheries Research Centre, Cairns, with a research program designed to produce fingerlings for a river and estuary recreational fishing restocking program (22) (Figure 1.3). The first commercial farm was established near Innisfail, North Queensland, in 1986. Since these early days, Barramundi aquaculture in now well-established in every state of Australia except Tasmania and total production has increased significantly to over 4,000 tonne annually.

1.1.4 Wild caught commercial fisheries production

In addition to aquaculture, Australia has a significant fisheries sector, which landed 162,762 tonne of product valued at $1.31 billion in 2010/11 (2). Major sectors of this industry include the Northern Prawn Fishery, the Eastern Tuna and Billfish Fishery, the South Bluefin Tuna Fishery, and the Southern and Eastern Scalefish and Shark Fishery (trawl sector and gillnet, hook and trap sector) (2).

Figure 1.4: Drawings of wild fish studied in this project; (A) Jack mackerel, *Trachurus declivis*; (B) Sand flathead, *Platycephalus bassensis*; (C) Tiger flathead, *Neoplatycephalus richardsoni* (Picture credit: www.fishbase.org).

The commercially important fish species sand flathead and tiger flathead (human consumption), and jack mackerel (bait and fishmeal production) (Figure 1.4) are all fished within Tasmanian waters as part of the Southern and Eastern Scalefish and Shark fishery (trawl sector) and the Small Pelagic fishery.

1.2 WHAT IS EPITHELIOCYSTIS?

Epitheliocystis is a bacterial condition that affects the gills and sometimes the skin of finfish. First described in common carp (*Cyprinus caprio*) in 1920 (23), this condition occurs in both wild and farmed fish populations and is currently known to affect over 80 different species of marine and freshwater fish (24-27).

Species of fish that have been reported as infected with epitheliocystis are taxonomically diverse, belonging to many different families including [a] Triakidae: leopard shark (*Triakis semifasciata*) (28); [b] Acipenseridae: white sturgeon (*Acipenser trasmonanus*) (28); [c] Clupeidae: Pacific herring (*Clupea pallasi*) (29) and pilchard (*Sardinops sagax*) (30); [d] Ictaluridae: brown bullhead (*Ictalurus nebulosus*) (31) and channel catfish (*Ictalurus punctatus*) (32); [e] Salmonidae: chum salmon (*Oncorhynchus keta*) (29), coho salmon (*Oncorhynchus kisutch*) (33), pink salmon (*Oncorhynchus gorbuscha*) (29), Chinook salmon (*Oncorhynchus tshawytscha*) (29), rainbow trout (*Oncorhynchus mykiss*) (34), Atlantic salmon (*Salmo salar*) (33), Arctic charr (*Salvelinus aplinus*) (35) and lake trout (*Salvelinus namaycush*) (36); [f] Syngnathidae: leafy seadragon (*Phycodurus eques*) (37) and greater pipefish (*Sygnathus acus*) (38); [g] Moronidae: sea bass (*Dicentrarchus labrax*) (39), white perch (*Morone americanus*) (40) and striped bass (*Morone saxatilis*) (40); [h] Terapontidae: silver perch (*Bidyanus bidyanus*) (41); [i] Carangidae: amberjack *(Seriola dumerili*) (5), yellowtail kingfish (*S. lalandi*) (17), yellowtail (*S. mazatlana*) (4), and jack mackerel (*Trachurus declivis*) (42); [j] Pomacentridae: white-ear scalyfin (*Parma microlepsis*) (43) and Tetraodontidae: tiger puffer (*Takifugu rubripres*) (44) (see Appendix S1 for complete list).

Epitheliocystis infections although often reported as benign, can cause a proliferative host response to the cysts. When a proliferative host response occurs, affected fish may appear to be lethargic, have increased mucus production and increase their ventilation

due to respiratory distress (24, 35, 45). Under light microscopy, spherical cysts within infected and hypertrophied gill lamellae tissue will mostly exhibit themselves as densely packed, appear basophilic when stained (with H&E) and may or may not be granular (25, 45-47). The cysts are surrounded by an inclusion membrane, which often thickens as the size of the inclusion increases and the cell nucleus, while still present, is distorted and pushed to the periphery of the cell wall (27, 35, 45, 48-50). Epitheliocystis inclusions vary in density and are often unevenly distributed throughout the gill filaments (27, 51). In severe cases of hyper-infection, such as in sea bream (*Sparus aurata*), the cyst-like intracellular lesions in the gill lamellae resulted in hyperplastic epithelial cells aggregated in layers around the cysts causing fusion of the infected lamellae and an infiltration of macrophages and eosinophils (52).

Most commonly associated with larval and juvenile fish, epitheliocystis can also affect adult fish. While many of the reported losses in aquaculture attributed to epitheliocystis have occurred in the larval or juvenile stage (26, 53, 54), the condition has been associated with mortalities of adults, such as farmed Arctic charr and yellowtail kingfish (25, 35). Under what circumstances mortalities occur however has yet to be determined.

Epitheliocystis has had an interesting history. Over the years the condition has been attributed to unicellular algae (23), protozoa (55), members of the Bedsonia group (40), *Rickettsia*-like organisms (56), and *Chlamydia*-like organisms. It is now known that epitheliocystis is caused by obligate intracellular, Gram-negative bacteria, taxonomically belonging to the order *Chlamydiales* as confirmed by PCR molecular characterisation and DNA sequencing (24, 26, 28, 57).

Research into this condition has increased in recent years, especially into the detection of the causative agent(s) using molecular techniques. Currently, twelve partial 16S rRNA gene sequences amplified by PCR and sequenced have been reported; all have been registered with GenBank (Table 1.1). Established primer pair 16SIGF and 16SIGR were developed to target the variable region of the *Chlamydiales* 16S gene (now known as the 'signature sequence' region) (58) and have since been used by most of the subsequent studies listed in Table 1.1.

Table 1.1: Available 16S rRNA sequences registered with NCBI GenBank at the start of 2011.

Species	Geographic Origin (Environment)	Tissue type	Gene (Accession No.)	Name	Length (bp)	Confirmation by histology	Reference
Atlantic Salmon, (*Salmo salar*)	Ireland (C) Norway (C)	Formalin-fixed gills	16S rRNA (AY462243-44)	*Ca.* Piscichlamydia salmonis	1,487	+ve	(57)
Barramundi, (*Lates calcarifer*)	Australia (C)	Frozen whole gills	16S rRNA (AY013474)	CRG98	215	+ve	(24)
Leafy sea dragon, (*Phycodurus eques*)	Australia (Cap)	Paraffin-embedded gills	16S rRNA (AY013396)	CRG20	224	-ve	(24)
Silver perch, (*Bidyanus bidyanus*)	Australia (C)	Paraffin-embedded gills	16S rRNA (AY013394)	CRG18	214	+ve	(24)
Arctic charr, (*Salvelinus alpinus*)	Canada (C)	Fresh gill samples	16S rRNA (Not submitted)	*Neochlamydia* sp.	289	+ve	(35)
Brown trout (*Salmo trutta*)	Norway (W)	nd	16S rRNA (EF153480)	uncultured *Chlamydiaceae* bacterium	1204	nd	Karlsen & Nylund (GenBank)
Atlantic salmon (*Salmo salar*)	Norway (C)	Karnovsky fixed gills	16S rRNA (DQ011662)	*Ca.* Clavichlamydia salmonicola	1294	+ve	(50)
Brown trout (*Salmo trutta*)	Norway (W)	Karnovsky fixed gills	16S rRNA (EF577392)	*Ca.* Clavichlamydia salmonicola	1294	+ve	(50)
Sharpsnout sea bream (*Diplodus puntazzo*)	Greece (C)	Formalin-fixed gills	nd	nd	nd	+ve	(26)
Atlantic Salmon, (*Salmo salar*)	Ireland (C)	Paraffin-embedded gills	16S rRNA (FN545849-52)	*Ca.* Clavochlamydia salmonicola	1244	+ve	(27)
Leopard shark, (*Triakis semifasciata*)	Switzerland (Cap)	Paraffin-embedded gills	16S rRNA (FJ001668)	UFC 1 Leopard Shark	294	+ve	(28)
Arctic charr, (*Salvelinus alpinus*)	North America (C)	Fresh gill samples	16S rRNA (GQ302987-8)	*Ca.* Piscichlamydia salmonis	263	+ve	(49)

nd – no data; C – cultured; Cap – captive; W – wild. ** This sequence was 100% identical to a *Neochlamydia* sp. isolated from a cat ocular sample (AY225593.1). The sample from Arctic charr has not been submitted to GenBank.

While not all of the host species were from aquaculture, the large majority were indicating that this condition may be playing a larger role in the health of fish on farms than previously thought. In addition, the diverse sequence structure of these CLOs has led to the opinion that this condition has highly host-specific causative agents.

1.3 *CHLAMYDIA* AND CHLAMYDIAL TAXONOMY

Chlamydia and *Chlamydia*-like bacteria as members of the Order *Chlamydiales* are obligate intracellular pathogens of many important medical and veterinary diseases, including trachoma, foetal abortions and respiratory infections (58, 59). They have an extremely diverse host range that varies from mammals (including humans, ovines, bovines, equines and fruitbats), avians, reptiles, bony and cartilaginous fishes, arthropods to free-living amoebae (28, 59-62).

Chlamydiae exhibit a unique two-stage developmental cycle that rotates between an infectious elementary body (EB) that resides within the cytoplasm and an intracellular vegetative reticulate body (RB) that replicates through binary fission (58, 60). Variations of this developmental cycle have been reported, including the crescent body for members of the family *Parachlamydiaceae* (63), and the cycle between primary long cells and small cells (47).

The taxonomy of this interesting bacterial group has changed significantly over the past two decades (Figure 1.5). In the early 1990s, the phylogeny of the order *Chlamydiales* included only one family (*Chlamydiaceae*), with one genus (*Chlamydia*) and four described species (*C. trachomatis*, *C. pecorum, C. pneumoniae* and *C. psittaci*) (Figure 1.5 A). Towards the end of the 1990s, molecular techniques were becoming more common in research which resulted in an increased uptake of gene sequencing, changing how we described new species. As a result, a review of the taxonomy of the order *Chlamydiales* was published in 1999 which described new rules of classification for species within the order *Chlamydiales* based on their sequence similarity (58). The authors stated that for any future bacterial isolate or sequence to be included within the order *Chlamydiales* it required a ≥ 80 % sequence similarity of the 16S rRNA gene to the known and accepted members already within the order. It also set out that a ≥ 90 % sequence similarity would confer the same family, ≥ 95 % sequence similarity would confer the same genus

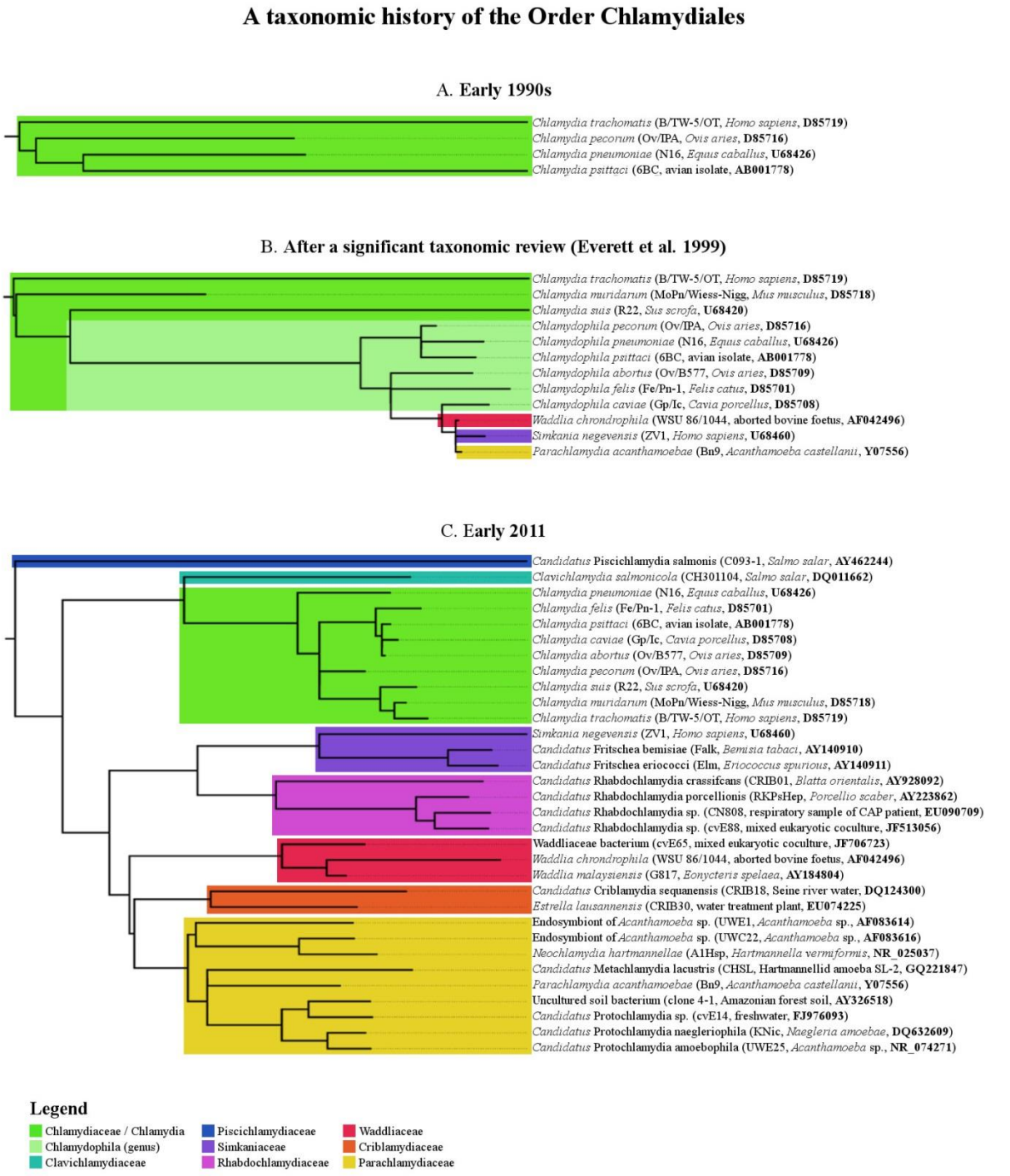

Figure 1.5: A taxonomic history of the Order *Chlamydiales* showing the change in diversity over time; a) Taxonomy in early 1990's (dataset trimmed to 1476 bp), b) Everett's review 1999 (dataset trimmed to 1485 bp), c) taxonomy at end of 2010 (dataset trimmed to 1346 bp). Trees were constructed by performing a maximum-likelihood analysis (GTR+G+I model) using MEGA5 (64) on each aligned and trimmed dataset. Strain, host and GenBank accession no. are given in brackets.

and ≥ 97 % would confer the same species (58). Following these rules, the diversity of the order *Chlamydiales* increased, with three newly described type species with their own novel family lineage (*Waddliaceae*, *Simkaniaceae* and *Parachlamydiaceae*). In addition, a split in the *Chlamydiaceae* family occurred with two genera (*Chlamydia* and *Chlamydophila*) across nine species (Figure 1.5 B). Another decade later and the diversity of the Order *Chlamydiales* again increased substantially. Along with the now recognised four families, there is an additional four *Candidatus* families (*Ca*. Rhabdochlamydiaceae, *Ca*. Criblamydiaceae, *Ca*. Piscichlamydiaceae and *Ca*. Clavichlamydiaceae) within the order (Figure 1.5 C). Importantly, the split that occurred within the *Chlamydiaceae* was reunited after further molecular evidence placed all species within the genus *Chlamydia*.

Currently, there are two family lineages with two type species of Chlamydial pathogens sequenced and an additional six partial sequences available from fish (Table 1.1). To date none of these CLOs have been isolated and cultured in pure form. In addition, the bacteria have been sourced from Atlantic salmon of both marine (*Ca*. Piscichlamydia salmonis) and freshwater (*Ca*. Clavichlamydia salmonicola) origins (50, 57).

The novel lineages that have *Candidatus* status reflect those organisms that have had their 16S rRNA gene sequenced to nearly full-length (between 1300 – 1500 bp). There are however, hundreds of partial sequences (between 250 – 750 bp) registered with GenBank, which a significant proportion may, after further analysis, also be novel species within the order. Phylogenetic analysis of these novel lineages reveals a richer diversity within the order *Chlamydiales* than previously thought.

1.4 PROVING CAUSATION

The Henle-Koch Postulates (known as Koch's Postulates), were developed for evaluating the causal relationship between clinical disease and their aetiological agent(s) (Table 1.2). These postulates were not full-proof however, and over the years there have been many cases where they could not be fulfilled satisfactorily. Limitations on both 'the microorganisms causing disease anew when introduced into a healthy organism' (point 3, table 1.2) and more importantly for this study, with 'isolation of the agent from a diseased organism' (point 2, table 1.2) (65) have been a problem for many disease

researchers trying to prove causation. In addition, these postulates are not applicable to all pathogenic bacteria as they do not take into consideration; the asymptomatic carrier status of bacteria, the biological spectrum of the disease, what epidemiological elements are factored into causation, multiple infections, or a single syndrome having multiple causes under different conditions (66).

Table 1.2: Koch's Postulates for disease causation

1. The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms
2. The microorganism must be isolated from a diseased organism and grown in pure culture
3. The cultured microorganism should cause disease when introduced into a healthy organism
4. The microorganism must be re-isolated from the inoculated, diseased experimental host and identified as being identical to the original species causative agent

Bacteria belonging to the Order *Chlamydiales* (as described above), are intracellular bacteria that require a host cell to survive. Because of this requirement, research has been somewhat limited as the *in vitro* methods are rather finicky. While there are established methods for culture of the traditional *Chlamydia* and some CLOs within both cell lines and through amoebal co-culture (60), there are currently no available *in vitro* methods for any of the fish CLOs, although many have been tried (Table 1.3).

In the absence of an *in vitro* protocol for the purification of *Chlamydia*-like agents associated with epitheliocystis, another route needed to be taken to prove causal relationship between CLOs and epitheliocystis. With the increase in technology and more specifically, with the increasing uptake of DNA amplification and sequencing, a set of molecular postulates were developed in 1996 (Table 1.4) (67). This set of guidelines for establishment of causal relationships between disease and the aetiological agent were much broader and tried to alleviate the issues that arose with Koch's Postulates by taking a much more holistic approach that included among others, known biological characteristics and relevant epidemiology.

Table 1.3: Unsuccessful reports of *in vitro* culture of epitheliocystis aetiological agent.

Species	Origin		Histology		*In vitro* Method	Ref
Bluegill (*Lepomis macrochirus*)	USA (C)	+ve	Enlarged, basophilic inclusion bodies present	Cell culture	Tissue homogenates inoculates onto RTg-2 (fibroblast cell line from rainbow trout) cell line	(55)
Lake trout (*Salvelinus namaycush*)	USA (C)	+ve	Basophilic inclusions distributed along lamellae with marked epithelial hyperplasia and hypertrophy	Cell culture	Tissue homogenates inoculated onto EPC (epithelioma papillosum cyprinid) and McCoy cell lines	(36)
Threespine sickleback (*Gasterosteus aculeatus*) Fourspine sickleback (*Apeltes quadracus*)	Canada (W)	+ve	Nodules appear as a swirled, basophilic mass surrounded by hyperplastic epithelium	Bacterial culture	Epitheliocystis nodules were squashed between glass slides and plated onto BHI (brain-heart infusion) agar and blood agar, both with +2% NaCl	(68)
Yellowtail (*Seriola lalandi**)	Ecuador (C)	+ve	Enlarged cells densely packed with basophilic granules found within gill and skin epithelium	Bacterial culture	Early juvenile homogenate inoculated onto marine agar and TCBS	(17)
Grass carp (*Ctenopharyngodon idella*)	Austria (C)	+ve	Proliferative lesions with granulated bacterial inclusions	Cell culture	Homogenised epitheliocystis positive gill filaments were inoculated onto EPC-57 (epithelioma papulosum cyprini, CCB-816 (common carp brain) and FHM-57 (flathead minnow) cell lines	(51)

* formerly *S. mazatlana;* C – cultured; W – wild

Table 1.4: Fredricks and Relman's molecular postulates for disease causation (67).

1. A nucleic acid sequence belonging to a putative pathogen should be present in most cases of an infectious disease. Microbial nucleic acids should be found preferentially in those organs or gross anatomic sites known to be diseased and not in those organs that lack pathogens.
2. Fewer, or no, copy numbers of pathogen-associated nucleic acid sequences should occur in hosts or tissues without disease
3. With resolution of the disease, the copy number of pathogen-associated nucleic acid sequences should decrease or become undetectable. With clinical relapse, the opposite should occur.
4. When sequence detection predates disease, or sequence copy number correlates with severity of disease or pathology, the sequence-disease association is more likely to be a causal relationship.
5. The nature of the microorganism inferred from the available sequence should be consistent with the known biological characteristics of that group of organisms
6. Tissue-sequence correlates should be sought at the cellular level: efforts should be made to demonstrate specific *in situ hybridisation* of microbial sequence to areas of tissue pathology and to visible microorganisms or to areas where microorganisms are presumed to be located.
7. These sequence-based forms of evidence for microbial causation should be reproducible.

While there is a great need to develop an *in vitro* protocol for the culture of the causative agent(s) of epitheliocystis, which may lead to a fulfilment of Koch's postulates, this pathway is not feasible at this time. As a result, the molecular postulates developed by Fredericks and Relman were used to establish disease causation in this study.

1.5 Aims, Significance and Project Development

The species selected for this study were yellowtail kingfish, striped trumpeter and barramundi (farmed fish), and jack mackerel, sand flathead and tiger flathead (wild fish). The species were selected based on their importance to the seafood industry as well as the availability of samples (both new and archival).

The overall objective of this thesis was to study the disease Epitheliocystis in Australian finfish and to gain a greater understanding of the aetiological agent(s) involved. Currently, there is very little information available on the infection levels of epitheliocystis in both wild and farmed fish in Australia. In addition, the diversity of epitheliocystis agent(s) is not fully known and as it stands, no epitheliocystis agent has been characterised in the Southern Hemisphere.

To address these issues and the overall objective of this study the following specific aims were investigated:

a) Investigate and document infection levels in wild and farmed fish, and determine which factors affect epitheliocystis outbreaks
b) Characterise epitheliocystis agents from at least two species of Australian fish
c) Critically review the current understanding of the role of *Chlamydia*-like organisms found in association with epitheliocystis

1.6 THESIS STRUCTURE

In total this thesis contains 7 chapters and is structured as follows;

- This chapter (Chapter 1) is a general introduction to aquaculture, the species studied, what epitheliocystis is and its relationship with *Chlamydia*-like organisms and ends with the project aims
- Chapter 2 describes the results from a wild fish survey undertaken in the waters of Eastern Tasmania.
- Chapter 3 describes and characterises epitheliocystis infections and the causative bacterial agent from yellowtail kingfish, *Seriola lalandi*, from farms in South Australia.
- Chapter 4 describes and characterises epitheliocystis infections and the chlamydial agents from striped trumpeter, *Latris lineata*, held in recirculating tanks in Hobart, Tasmania and compared with wild fish caught from Southwest Tasmania.
- Chapter 5 describes and characterises epitheliocystis infections and the chlamydial agents from 6 consecutively hatched cohorts and a cohort of pre-hatch eggs of farmed barramundi, *Lates calcarifer*, from a farm in South Australia.

- Chapter 6 is a critical review of the status of *Chlamydia*-like organisms that are found in association with epitheliocystis in fish, provides an update on the current knowledge of the taxonomy and diversity of chlamydial pathogens of fish, discusses the impact of these infections on the health of fish and highlights further areas of research required to understand the biology and epidemiology of epitheliocystis aetiological agents.
- Chapter 7 is a summary of the findings of this project and their significance and contribution to the known literature.

CHAPTER 2 EPITHELIOCYSTIS IN THREE WILD FISH SPECIES IN TASMANIAN WATERS

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Keywords: epitheliocystis, jack mackerel, sand flathead, tiger flathead, water temperature, wild finfish

2.1 INTRODUCTION

Epitheliocystis is a *Chlamydia*-like, intracellular bacterial condition that affects the gills and skin of finfish (for review see Nowak and LaPatra, 2006). It is characterised by the presence of membrane-enclosed basophilic inclusions, resulting in hypertrophy of host cells. Although epitheliocystis is usually benign, it may lead to inflammation in the gills. Increased mucus production and respiratory distress can be observed in infected fish (31, 46, 69).

This condition occurs in both wild and farmed fish populations and is currently known to affect over 80 different species of marine and freshwater fish (25, 70). Species of Australian fish that have been affected by epitheliocystis are cultured barramundi, *Lates calcarifer* (Bloch) (71), yellowtail kingfish, *Seriola lalandi* Valenciennes (17), silver perch, *Bidyanus bidyanus* (Mitchell) (41), Atlantic salmon, *Salmo salar* L. (72) and striped trumpeter, *Latris lineata* (Forster) (73); and wild caught rock cale, *Crinodus lophodon* Günther (74), red morwong, *Cheilodactylus fuscus* (Castelnau) (74), pilchard, *Sardinops sagax* (Jenyns) (30), white ear parma, *Parma microlepis* Günther (43) and jack mackerel, *Trachurus declivis* (Jenyns) (42). Epitheliocystis has been described in only one other platycephalid species, cultured bartail flathead *Platycephalus indicus* (L.)

from Japan (75). In this species, mass mortalities in the juvenile stage occurred and were associated with epitheliocystis infection.

There are several risk factors that have been reported to affect epitheliocystis infections, including water temperature. Both higher water temperatures (in Atlantic salmon (72)) and lower water temperatures (in greater amberjack *Seriola dumerili* (Risso) (5)) have been associated with increased infection levels. The present study aimed to investigate and document infection levels of epitheliocystis in three species of commercially important wild fish. Fish were surveyed for epitheliocystis infections of five separate occasions over a period of 11 months. The results presented here report on the prevalence and intensity of epitheliocystis in jack mackerel, sand flathead, *Platycephalus bassensis* Cuvier and tiger flathead, *Neoplatycephalus richardsoni* Castelnau found in eastern Tasmania waters.

2.2 Materials & Methods

Target species, jack mackerel, sand flathead and tiger flathead were opportunistically sampled from benthic trawling activities in the waters east of Flinders Island, and east of Freycinet Peninsula, Tasmania. A total of 575 fish were sampled over five sampling periods (December 2011, April 2012, July 2012, September 2012 and November 2012). For each sampling period, *c*. 40 fish were sampled from each of the target species. Water temperature and GPS coordinates were recorded for each trawl. Weight and length measurements were taken. The second gill arch on the sinistral side was sampled and fixed in 10% neutral buffered formalin for histology.

Samples were trimmed and routinely processed for histology. Gills were sectioned at 5 µm and stained with haematoxylin and eosin. Sections were examined using light microscopy to identify epitheliocystis inclusions and associated lesions.

Results from the visual inspection of H&E stained sections were quantified as a prevalence (expressed as a percentage) of fish affected and intensity (cysts per section/filaments per section). Associated pathological signs were recorded but not quantified. Data for different species for prevalence and length were pooled for each sampling time, and because the distribution of the data was non-normal (even after

transformations), the nonparametric Kruskal-Wallis test with post-hoc comparisons was used. A Spearman's rank correlation test was used to identify if a significant relationship existed between seawater temperature and the prevalence of epitheliocystis.

2.3 RESULTS

Mean length, mean weight and prevalence for each species at each sampling period is summarised in Table 2.1. There was a significant difference of fish length between the sampling periods ($KW = 41.156$, $df = 4$, $p < 0.001$), with the larger fish being sampled during July 2012, September 2012 and November 2012 (Table 2.2). Epitheliocystis was present in all three species in most sampling periods, with the exception of jack mackerel in December 2011 and April 2012, and sand flathead in December 2011. Epitheliocystis prevalence ranged from 0 – 20% in jack mackerel and sand flathead and 7.5 – 20% in tiger flathead. The sampling period had a statistically significant effect on the prevalence of epitheliocystis ($KW = 20.310$, $df = 4$, $p < 0.001$), with the greatest prevalence during the July 2012, September 2012 and November 2012 sampling periods, when seawater temperature was at its lowest (12.2 ($\pm$0.02), 13.8 ($\pm$0.01) and 16.4 (0.02), respectively) (Table 2.2). The sampling period had a statistically significant effect on the intensity of epitheliocystis ($KW = 20.211$, $df = 4$, $p < 0.001$), with the greatest intensity following the same pattern as prevalence (Table 2.2). In addition, the prevalence of epitheliocystis was negatively correlated with the seawater temperature (ranging from 13.6 – 19.8^{o}C) and this relationship was significant ($r = -0.160$, $p < 0.001$).

Membrane-enclosed cysts were filled with a basophilic material that was not always granular. These inclusions both occurred with (Figure 2.1 A, B) and without (Figure 2.1 C, D) a proliferative host response. Most jack mackerel and sand flathead that were epitheliocystis positive had only one cyst within the gill arch, while tiger flathead often had multiple cysts (< 10) per gill arch. However, there was no difference in intensity of epitheliocystis between the sampling periods for tiger flathead (data not shown). There was also no pattern to the location of the cysts, with cysts forming at the base, middle and tip of the lamellae.

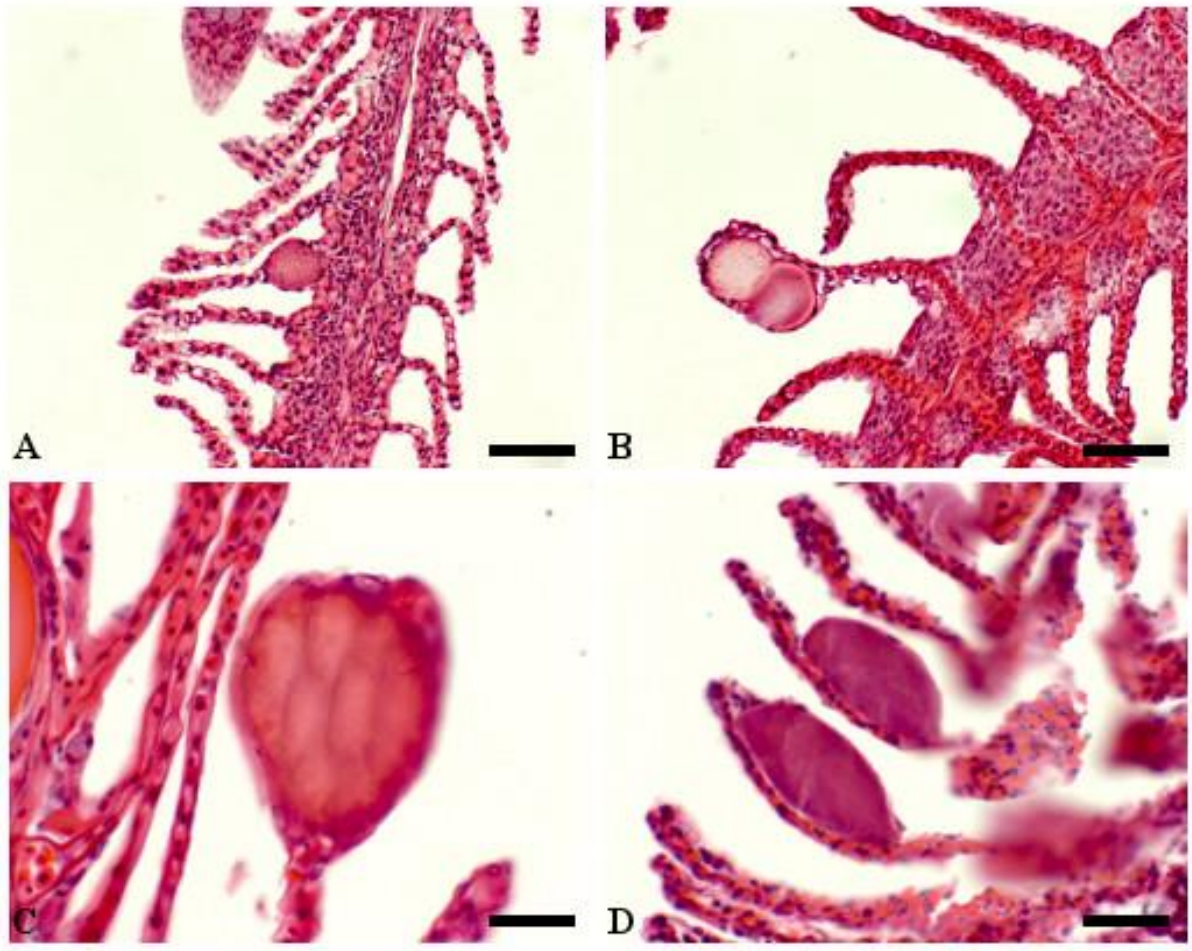

Figure 2.1: Gills showing membrane-enclosed epitheliocystis cysts (H&E staining). (A) Epitheliocystis with cellular host response along the primary lamellae in sand flathead (scale bar = 50 µm). (B) Double-cyst within the epithelium of the gill lamellae in tiger flathead (scale bar = 50 µm). (C) High magnification of basophilic inclusion at the tip of lamellae with no host response in tiger flathead (scale car = 25 µm). (D) Basophilic granular epitheliocystis cysts within the epithelium of the lamellae with no host response in jack mackerel (scale bar = 25 µm).

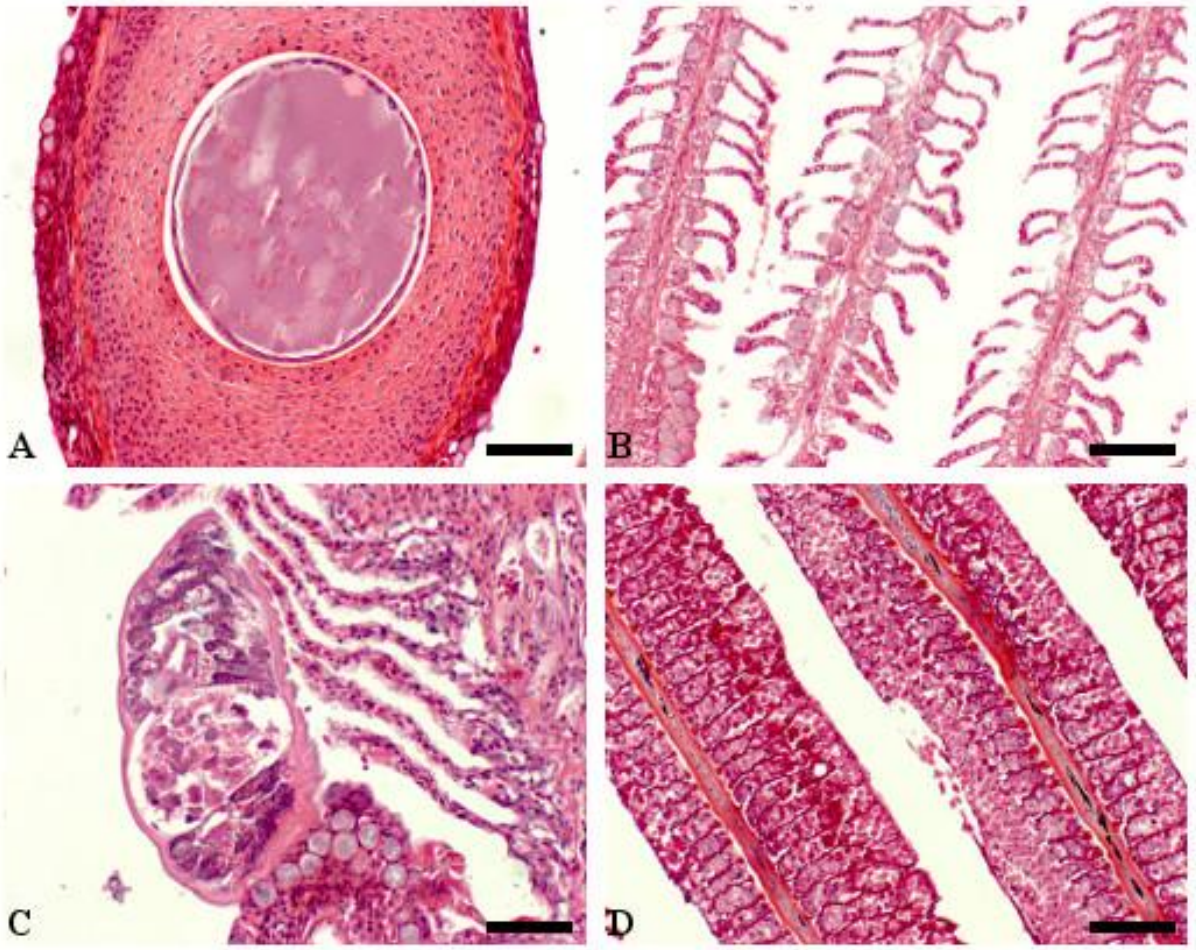

Figure 2.2: Other pathological changes and parasites found in the gills of jack mackerel, sand flathead and tiger flathead. (A) Large cyst of unknown aetiology within the central venus sinus in tiger flathead (scale bar = 50 µm). (B) Hypertrophic and hyperplasic mucus cells along the filament in sand flathead (scale bar = 100 µm). (C) Monogenean gill fluke attached to gill lamellae and causing cellular response in sand flathead (scale bar = 50 µm). (D) An unknown aetiology causing cellular proliferation and lamellar fusion in jack mackerel (scale bar = 100 µm).

In addition to the epitheliocystis, a variety of parasites, hypertrophic mucus cells, and other unknown aetiology conditions was observed within the gill arches of all three species (Figure 2.2). Large cysts of unknown aetiology which contained eosinophilic material were occasionally seen within the central venus sinus of tiger flathead only (1.5%) (Figure 2.2 A). Hypertrophic and hyperplastic mucus cells were observed regularly in sand flathead gills (18.4%), however this was seen in only two individuals of tiger flathead (1%) and did not occur at all in jack mackerel (0%) (Figure 2.2 B). Monogenean gill flukes were seen in all three species (jack mackerel 2.4%, sand flathead 18.4% and tiger flathead 20.2%) (Figure 2.2 C). Co-infections of epitheliocystis and parasites occurred in only 13.4% of cases and these co-infections were seen in sand flathead and tiger flathead only. Cellular proliferation and lamellar fusion was seen twice in jack mackerel (1.2%) (Figure 2.2 D).

2.4 Discussion

This is the first report of epitheliocystis in sand flathead and tiger flathead, adding to the ever increasing number of fish species affected by this condition. The infection levels of one cyst per gill arch reported here for jack mackerel, sand flathead and tiger flathead are consistent with a very low infection level (72). The bacterial infection occurred as large cysts within the epithelial cells along the filamentous lamellae. The site of this infection is quite common among fish infected with epitheliocystis and has occurred in other wild caught fish, such as Atlantic cod, *Gadus morhua* L. (46), brown bullhead, *Ameiurus nebulosus* (Lesueur) (31), greater pipefish, *Syngnathus acus* L. (38), Connecticut striped bass, *Morone saxatilis* (Walbaum), and white perch *Morone americanus* (Gmelin) (40). The varied proliferative and non-proliferative host reaction has occurred before in sea bream, *Sparus aurata* Linneaus. This reaction was due to the two different developmental cycles that epitheliocystis follows and it was suggested that fish age and environmental conditions play a role in these different developmental cycles (47).

The sampling period had a significant effect on the prevalence of epitheliocystis, which was greater during the colder sampling periods. The sampling period also had a significant effect on the fish length, with larger fish being sampled during the colder periods. The data from this study indicate that epitheliocystis prevalence is higher in

larger fish and during colder seawater temperatures, however it is impossible to separate between the effects of these two factors. A similar result was reported for epitheliocystis infections in winter flounder *Pleuronectes americanus* (Walbaum), where temperature and age could not be separated (76). It must be considered however, that while the seawater temperature data are limited, there was a significant correlation of increased epitheliocystis prevalence with decreased water temperature. Prevalence of epitheliocystis from cultured Spanish amberjack, *Seriola dumerili* and Connecticut striped bass also increased with decreasing water temperatures (5), however the opposite was true for Australian Atlantic salmon where increased prevalence was linked to increasing water temperatures (72).

The maximum prevalence reported of 20% for all three species is mid-range for wild fish previously reported with epitheliocystis. In particular, the prevalence of epitheliocystis in Jack mackerel is consistent with that previously reported (21%) (42). Comparatively, wild British Columbian species Pacific cod, *Gadus macrocephalus* Tilesius and Pacific tomcod, *Microgadus proximus* (Girard), were on the lower end of the prevalence range (1 and 2.5%, respectively), while rougheye rockfish, *Sebastes aleutianus* (Jordan & Evermann) and canary rockfish, *Sebastes pinniger* (Gill) were on the upper end of the prevalence range (61 and 67%, respectively) (29).

Monogenean parasites were found in all three target species. These parasites have been reported previously in sand flathead and jack mackerel, both sampled from Tasmanian waters (42). These monogeneans are host-specific and therefore it is highly likely that three different species of these parasites were observed. Although co-infections of epitheliocystis with monogenean parasites have previously been reported (10), the low occurrence of co-infections indicates that parasites had a negligible effect on epitheliocystis infection rates.

The large cysts of unknown aetiology that stained basophilic, had pink globules and were found within the central venus sinus in the tiger flathead have been seen previously in jack mackerel and red cod, *Pseudophycis bachus* (Forster) (42, 77), which were also sampled in Tasmania. In addition, similar large cyst-like structures have been observed in the kidney of several rockfishes, *Sebastes* spp. (78) and the cyst-like

Table 2.1: Mean length, mean weight and prevalence of epitheliocystis in the target species, Jack mackerel, Sand flathead and Tiger flathead

	Jack mackerel				Sand flathead				Tiger flathead			
Sampling time	***n***	**Length (mm) mean (SE)**	**Weight (g) mean (SE)**	**Prevalence % (*n* fish +ve)**	***n***	**Length (mm) mean (SE)**	**Weight (g) mean (SE)**	**Prevalence % (*n* fish +ve)**	***n***	**Length (mm) mean (SE)**	**Weight (g) mean (SE)**	**Prevalence % (*n* fish +ve)**
December 2011	51	179.40 (5.8)	100.5 (7.4)	0 (0)	39	331.0 (6.9)	352.5 (19.2)	0 (0)	40	339.3 (10.5)	401.1 (30.5)	7.5 (3)
April 2012	40	170.5 (5.2)	86.3 (3.3)	0 (0)	40	351.5 (9.0)	344.1 (33.8)	5 (2)	40	327.5 (13.1)	287.4 (35.0)	7.5 (3)
July 2012	5	204.0 (4.3)	107.0 (5.8)	20 (1)	40	352.3 (8.7)	347.0 (32.7)	20 (8)	40	330.9 (8.8)	284.0 (24.2)	7.5 (3)
September 2012	40	182.8 (2.9)	80.9 (3.4)	5 (2)	40	337.1 (6.6)	293.3 (20.9)	20 (8)	40	371.0 (9.5)	387.0 (26.0)	20 (8)
November 2012	30	201.2 (3.1)	103.0 (3.8)	3 (1)	47	377.7 (8.6)	411.3 (30.0)	19 (9)	43	380.7 (11.7)	456.2 (48.5)	14 (6)

Table 2.2: Pooled species data used for statistics

Sampling time	**Sea temp* mean (SE)**	***n***	**Length (mm) mean (SE)**	**Prevalence (%) mean (SE)**	**Intensity mean (SE)**
December 2011	17.7 (0.02)	130	274.1 (8.0)^{A}	2.3 (1.3)^{A}	0.0008 (0.0006)^{A}
April 2012	19.5 (0.03)	120	283.2 (9.2)^{A,B}	4.2 (1.8)^{A}	0.0028 (0.0015)^{A}
July 2012	14.2 (0.02)	85	333.5 (6.9)^{C}	14.1 (3.8)^{B}	0.2505 (0.2448)^{B}
September 2012	13.8 (0.01)	120	297.0 (8.5)^{B}	15.0 (3.3)^{B}	0.0269 (0.0195)^{B}
November 2012	16.4 (0.02)	120	334.6 (8.9)^{C}	13.3 (1.2)^{B}	0.0109 (0.0042)^{B}

*Sea temperature (^{o}C)

Superscripts denote significant differences between sampling periods

structure in bluestripe snapper, *Lutjanus kasmira* (Forsskål) (79). Following DNA sequencing, the authors determined that these cysts were shown to contain *Chlamydia*-like bacteria (Corsaro and Work, 2012). We did not examine internal organs of the wild fish, however in bluestripe snapper these cysts were absent from the gills (Corsaro and Work, 2012).

The causative agent of epitheliocystis previously reported in jack mackerel (42) has not been determined as *Chlamydia*-like or otherwise, due to lacking ultrastructural and molecular evidence. This investigation was outside the scope of this project. While many of the recently described agents of epitheliocystis have been *Chlamydia*-like, this is still to be confirmed for jack mackerel, sand flathead and tiger flathead.

2.5 Acknowledgements

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CHAPTER 3 MOLECULAR CHARACTERISATION OF "*CANDIDATUS* PARILICHLAMYDIA CARANGIDICOLA", A NOVEL *CHLAMYDIA*-LIKE EPITHELIOCYSTIS AGENT IN YELLOWTAIL KINGFISH, *SERIOLA LALANDI* (VALENCIENNES), AND THE PROPOSAL OF A NEW FAMILY, "*CANDIDATUS* PARILICHLAMYDIACEAE" FAM. NOV. (ORDER *CHLAMYDIALES*)

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3.1 ABSTRACT

Three cohorts of farmed yellowtail kingfish (*Seriola lalandi*) from South Australia were examined for *Chlamydia*-like organisms associated with epitheliocystis. To characterise the bacteria, 38 gill samples were processed for histopathology, electron microscopy, and 16S rRNA amplification, sequencing, and phylogenetic analysis. Microscopically, the presence of membrane-enclosed cysts was observed within the gill lamellae. Also observed was hyperplasia of the epithelial cells with cytoplasmic vacuolisation and fusion of the gill lamellae. Transmission electron microscopy revealed morphological features of the reticulate and intermediate bodies typical of members of the order *Chlamydiales*. A novel 1,393 bp 16S chlamydial rRNA sequence was amplified from gill

DNA extracted from fish in all cohorts over a 3-year period that corresponded to the 16S rRNA sequence amplified directly from laser-dissected cysts. This sequence was only 87% similar to the reported "*Candidatus* Piscichlamydia salmonis" (AY462244) from Atlantic salmon and Arctic charr. Phylogenetic analysis of this sequence against 35 *Chlamydia* and *Chlamydia*-like bacteria revealed that this novel bacterium belongs to an undescribed family lineage in the order *Chlamydiales*. Based on these observations, we propose this bacterium of yellowtail kingfish be known as "*Candidatus* Parilichlamydia carangidicola" and that the new family be known as "*Candidatus* Parilichlamydiaceae".

3.2 INTRODUCTION

The world's demand for seafood far exceeds the current supply (80), and significant increases in research and development of new species for aquaculture have been initiated in response to this demand. The yellowtail kingfish (YTK), *Seriola lalandi*, is an example of one such new aquaculture species, and for the last decade, efforts have focused on developing a commercial aquaculture industry for the species in Australia and New Zealand. Nine species are currently recognized in the genus *Seriola*, and three are cultured commercially around the world: *S. dumerili*, *S. quinqueradiata*, and *S. lalandi* (3, 6, 81). However, the establishment and commercial production of YTK aquaculture, although considered successful, has been beset by an increased incidence of disease within the cultured populations (10, 14, 82, 83).

The order *Chlamydiales* is a constantly evolving taxonomic classification, and in the early 1990s, it was considered to be a closely related group of only four species of bacteria, all contained within one genus. However, with the increased use of molecular techniques and phylogenetic analysis, the *Chlamydiales* have undergone considerable revision. In 1999, classification rules for *Chlamydiales* bacteria were reviewed, which subsequently resulted in significant changes in the taxonomic classification of organisms within the order (58). These proposed rules were based upon the 16S rRNA sequence similarity, and it was subsequently accepted that a sequence similarity of 95% would constitute a new genus, a sequence similarity of 90% would constitute a new family, and a sequence similarity of 80% would constitute a different order (58).

Chlamydia-like organisms (CLO) are pathogens that are known to cause disease and mortality in a wide range of species, including humans, sheep, cattle, koalas, bats, birds, insects, and, most recently, fish (60), including the cutaneous and branchial infection referred to as epitheliocystis (25). Although epitheliocystis is often benign, it can cause epithelial hyperplasia and inflammation of infected tissues, resulting in significantly reduced growth (46, 47). In severe cases of hyperinfection, the cyst-like branchial lamellar lesions can result in increased lamellar fusion with consequent respiratory distress and death (24, 35). Epitheliocystis outbreaks have been reported in many aquaculture species, including barramundi, *Lates calcarifer* (71); white sturgeon, *Acipenser transmontanus* (84); silver perch, *Bidyanus bidyanus* (41); Atlantic salmon, *Salmo salar* (53); red sea bream, *Pagrus major* (54); carp, *Cyprinus carpio* (85); and yellowtail kingfish, *S. lalandi* (10). It has been suggested that increased water temperature increases the risk of infection and the incidence of mortality, although additional experimental work is required (25). Many of the reported losses in aquaculture attributed to epitheliocystis occur in the larval or juvenile stage (25, 53, 54, 85). Although this condition has been reported for over 80 years, the causative agent or agents of epitheliocystis have yet to be successfully cultured in vitro. Only recently have three of the etiological agents of epitheliocystis been more completely described as belonging to the order *Chlamydiales*, a group of obligate intracellular bacteria sharing unique and complex developmental cycles (24, 27, 28, 50, 57, 86), whereas four additional organisms have been characterized using molecular methods and phylogenetic analyses (24, 28, 57).

The objective of this study was to identify, characterize, and compare *Chlamydia*-like 16S rRNA genetic sequence data isolated and amplified from epitheliocystis-affected gills of YTK over three different cohorts from commercial farms in South Australia and from archival material held at the University of Tasmania.

3.3 Materials & Methods

3.3.1 Ethics statement

Sampling of animals for this study was conducted opportunistically and after commercial harvest. Animals were killed by commercial staff and subject to standard industry harvest practices.

3.3.2 Sample collection

A total of 38 YTK, S. lalandi, from three cohorts were analysed. They were sampled during commercial harvests from sea cages located throughout the YTK commercial production zone in South Australia. Samples were taken from the following years: 2008 (n = 8), 2009 (n = 10), and 2010 (n = 20) cohort YTK. The second gill arch on the sinistral side was sampled and fixed in 10% neutral buffered formalin for histology, with a small subsample fixed in RNAlater (Epicentre, Wisconsin) for molecular testing. Archival samples from 2002 were used for the transmission electron microscopy (TEM) and laser-dissected cyst analyses (87.5% of kingfish from 2002 were epitheliocystis positive; infection was previously reported on the basis of histology (10)).

Fish sampled from the 2009 YTK cohort were approximately 3 kg in size and exhibited clinical signs that included heavy infection with the monogenean *Benedenia seriolae*, slow swimming, and poor condition. Gills were swollen, with shortened filaments, and white streaking was observed along the filaments. Fish from the 2010 YTK cohort were commercially harvested at a size of 3.5 kg at the time of sampling, and all fish appeared to be clinically healthy. Fish sampled from the 2008 YTK cohort were clinically healthy.

3.3.3 Histopathology

Formalin fixed gills were trimmed and routinely processed for histology. The gills were sectioned at 5 µm and stained with haematoxylin and eosin. The sections were examined by light microscopy to identify epitheliocystis inclusions and associated lesions.

3.3.4 Transmission electron microscopy

Selected areas of gill tissue from archival samples were procured from the paraffin blocks and deparaffinised in xylene overnight prior to rehydration through a graded ethanol series. Tissues were placed in Sorenson's phosphate buffer (0.1 M; pH 7.2) prior to fixation in Karnovsky's solution for 4 h at 4°C. Following a brief rinse in phosphate buffer, the tissues were post-fixed in 1% aqueous osmium tetroxide, dehydrated through a graded acetone series, infiltrated, and embedded in epoxy resin. Thin sections cut at 60 to 90 nm were stained with 4% uranyl acetate and lead citrate prior to

examination with a Philips EM 400 transmission electron microscope at 80 kV (Philips Electronic Instruments, Mahwah, NJ).

3.3.5 Laser dissection of epitheliocystis cysts

Paraffin-embedded blocks were sectioned and mounted unstained on polyethylene naphthalate (PEN) membrane slides. The slides were then examined, and cysts were cut using a Leica LMD 6500 microscope (Leica, Wetzlar, Germany). The laser-cut cysts were dropped by gravity into a PCR tube cap and were used for further processing.

3.3.6 DNA extraction

DNA was extracted from all cohorts tested and the laser-dissected cysts using a commercial DNA extraction kit (Epicentre MasterPure Complete DNA and RNA Purification Kit) according to the manufacturer's instructions. The DNA pellet was rinsed with 70% ethanol and resuspended in 100 µl of Tris-EDTA (TE) buffer.

3.3.7 *Chlamydiales*-specific 16S rRNA PCR amplification and sequencing

The presence of chlamydial DNA was confirmed by *Chlamydiales*-specific 16S rRNA PCR using primers 16SIGF (5'–CGG CGT GGA TGA GGC AT–3') and 16SIGR (5'–TCA GTC CCA GTG TTG GC–3'), resulting in a 298-bp signature sequence, as described previously (58). The amplification reaction was performed with initial denaturation (95°C; 10 min), followed by 35 cycles of denaturation (95°C; 30 s), annealing (54°C; 30 s), and extension (72°C; 1.5 min), and a final extension (72°C; 7 min). Distilled-H2O negative controls were performed in triplicate. For the expanded 800-bp 16S rRNA sequence from selected samples, the 16SIGF primer was matched with the 806R 16S rRNA primer (87). The amplification reaction was initiated by denaturation (95°C; 10 min), followed by 35 cycles of denaturation (95°C; 30 s), annealing (55°C; 45 s), and extension (72°C; 45 s) and a final extension (72°C; 7 min). For the nearly full-length 16S rRNA sequence, the 16SIGF primer was matched with the 16SBI primer (88). The amplification reaction was initiated by denaturation (95°C; 10 min), followed by 35 cycles of denaturation (95°C; 1 min), annealing (55°C; 1 min), and extension (72°C; 1.5 min) and a final extension (72°C; 7 min).

After each PCR, separation of PCR products by agarose gel electrophoresis was performed, followed by visualization of a band at the expected size by UV transillumination (254 nm). The amplified PCR products were purified using a PureLink PCR Purification Kit (Invitrogen) before being sent for sequencing at the Australian Genome Research Facility, Brisbane, Australia. Sequences were analysed with Geneious Pro (89). The identities of amplified 16S rRNA sequences were determined by the BLAST-n algorithm (90) against sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov).

3.3.8 Primer design, validation and PCR of laser dissected cysts

After the samples for the three cohorts were screened using the *Chlamydiales* primer pair (16SIGF/16SIGR), YTK-specific primers were designed to validate the specificity of the results. A sequence alignment was performed using the ClustalW alignment algorithm with the YTK sequence reported here and from additional *Chlamydiales* species obtained from GenBank. The resulting primers, YTKfor (5'–GGG CCT TGC GGA TCG T–3') and YTKrev (5'–CCG CTA CTC TCA AGT TC–3'), were designed to amplify a YTK epitheliocystis agent-specific 16S rRNA sequence with an expected PCR product size of 280 bp. The YTKfor/YTKrev primer pair was validated against known epitheliocystis-positive samples from other fish species (data not shown).

To confirm that the chlamydial DNA detected from the YTK gill sample was the same as that within the epitheliocystis cysts, 16S rRNA PCR of the laser-dissected cysts was performed using primers YTKfor and YTKrev. The amplification reaction performed was the same as the initial PCR screening reaction described above.

3.3.9 Molecular phylogenetic analysis

The partial 16S rRNA region sequenced for the taxon reported here and data from additional *Chlamydiales* species and out group taxa obtained from GenBank (see Appendix S2 in the supplemental material) were initially aligned using MUSCLE version 3.7 (91) with ClustalW sequence weighting and unweighted-pair group method using average linkages (UPGMA) clustering for iterations 1 and 2. The resultant alignment was refined by eye using MESQUITE (92). After the alignment of the 16S data set was edited,

the ends of each fragment were trimmed to match the shortest sequence in the alignment.

The software jModelTest version 0.1.1 (93, 94) was used to estimate the best nucleotide substitution models for this data set. Bayesian inference analysis of the 16S rRNA data set was performed using MrBayes version 3.1.2 (95) run on the CIPRES portal (96) to explore relationships among these taxa. Bayesian inference analysis was conducted on the 16S rRNA data set using the GTR + I + G model predicted as the best estimator by the Akaike information criterion (AIC) and Bayesian information criterion (BIC) in jModelTest. Bayesian inference analysis was run over 10,000,000 generations (ngen = 10,000,000) with two runs each containing four simultaneous Markov chain Monte Carlo (MCMC) chains (nchains = 4), and every 1,000th tree was saved (samplefreq = 1,000). Bayesian analysis used the following parameters: nst = 6, rates = invgamma, ngammacat = 4, and the priors parameters of the combined data set were set to a ratepr of variable. Samples of substitution model parameters and tree and branch lengths were summarized using the parameters "sump burnin = 3,000" and "sumt burnin = 3,000." These "burnin" parameters were chosen because the log likelihood scores "stabilized" well before 3,000,000 replicates in the Bayesian inference analyses.

Maximum-likelihood analysis was performed on the 16S data set using the RAxML algorithm (97) on the CIPRES portal with the gamma rate model of heterogeneity and maximum-likelihood search estimating the proportion of invariable site parameters. Nodal support was inferred based on 100 bootstrap replicates.

3.3.10 Epidemiology

The prevalence of CLO detected by PCR was calculated as a percentage of all samples tested for a particular cohort (Table 3.1). The prevalence and intensity of epitheliocystis infection in YTK was calculated after light microscopy examination of H&E-stained gill sections. Absolute counts of cysts and filaments for each fish were recorded. The mean cyst count and mean intensity ($Intensity = \frac{cysts\ per\ section}{filaments\ per\ section}$) were calculated. Statistics were conducted with the Tinn-R 2.3.7.1 statistical package (2001; GUI for R language and environment; https://sourceforge.net/projects/tinn-r/). A residual plot and Bartlett test were used to test the assumption of homogeneity of variances, and the

mean intensity variable was square root transformed to meet this assumption. A one-way analysis of variance (ANOVA) was performed, and a Tukey honestly significant difference (HSD) test was used to detect any differences between cohorts (Table 3.1).

3.3.11 Nucleotide sequence accession number

The 16S rRNA gene sequence of the YTK epitheliocystis agent is available on GenBank under the accession number JQ673516.

3.4 RESULTS

3.4.1 Histopathology

Epitheliocystis was present in fish from all three cohorts. Bacterial cysts manifested as membrane-enclosed granulated basophilic inclusions. These basophilic inclusions were not always associated with cellular proliferation, and the majority were at the base of the gill lamellae (Figure 3.1 A and B). In gills with evidence of epitheliocystis, histopathological changes included cellular hyperplasia and vacuolation of cells with lamellar fusion (Figure 3.1 C and D).

3.4.2 Transmission electron microscopy

Transmission electron microscopy of the epitheliocystis inclusions revealed that the organisms were tightly packed within the membrane-bound vacuole. These vacuoles contained elongated reticulate bodies (RBs) (Figure 3.2 A and B) and spherical intermediate bodies (IBs) (Figure 3.2 C and D). Head and tail bodies were observed in association with the inclusions (Figure 3.2 C), although elementary bodies (EBs) were not observed. The RBs ranged in size from 83 by 211 nm to 100 by 361 nm, while the IBs ranged in size from 353 by 470 nm to 924 by 941 nm.

3.4.3 Epidemiology of novel YTK *Chlamydia*-like organism

The prevalence of infection based on the results from two methods (PCR and histopathology) varied greatly. PCR proved much more sensitive for detection of epitheliocystis, with cohort positivity ranging from 80 to 100% for PCR compared to 20 to 100% for histopathology (Table 3.1). Significant differences in the mean intensities of epitheliocystis inclusions were observed between the 2008 and 2009–2010 cohorts only ($F = 64.702$; $df = 2,116$; $p < 0.001$) (Table 3.1).

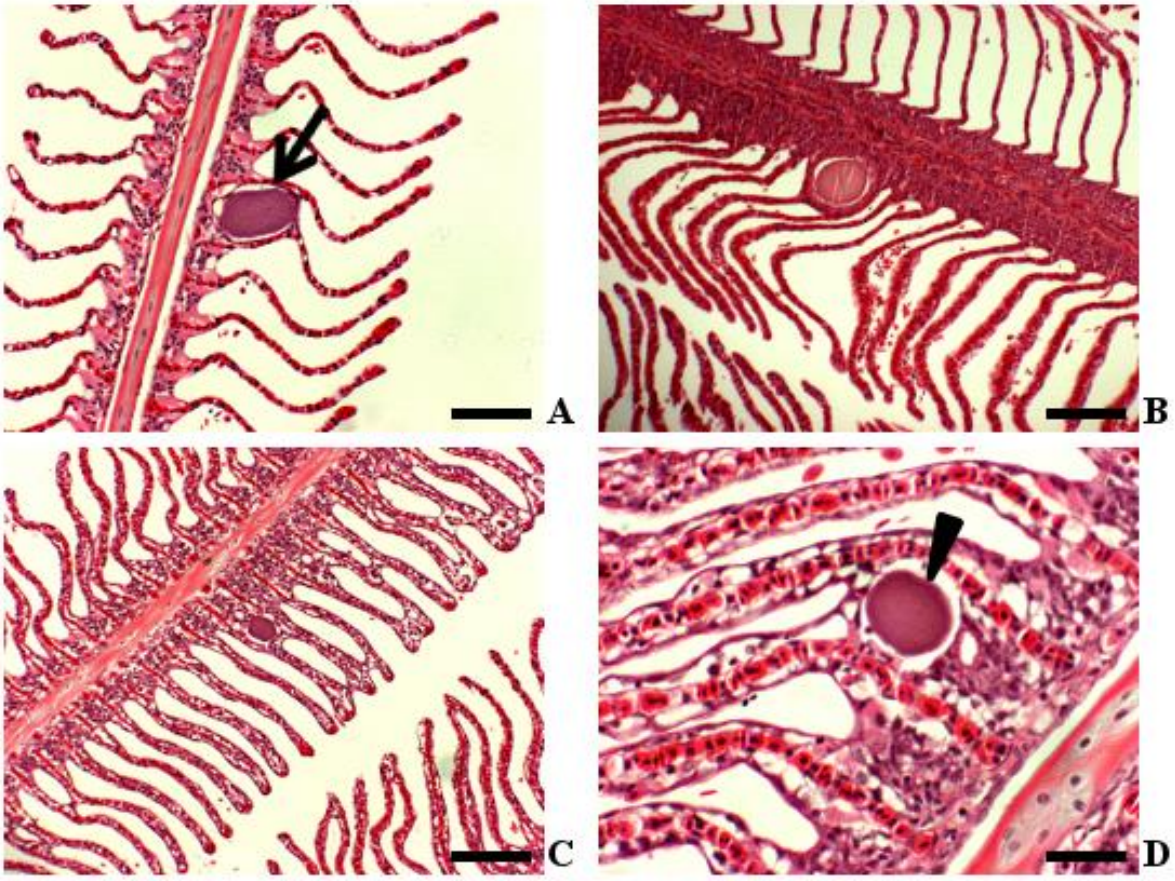

Figure 3.1: Yellowtail kingfish gills showing epitheliocystis (H&E staining). (A) Basophilic inclusion with no associated host response (scale bar = 100 µm) (2008 cohort). (B) Membrane-enclosed inclusion at base of lamellae (scale bar = 100 µm) (2010 cohort). (C) Basophilic inclusion with an associated lamellar fusion (scale bar = 200 µm) (2008 cohort). (D) Higher magnification of basophilic inclusion at base of lamella associated with proliferation and vacuolation of the basal lamellar epithelium (scale bar = 50 µm) (2009 cohort).

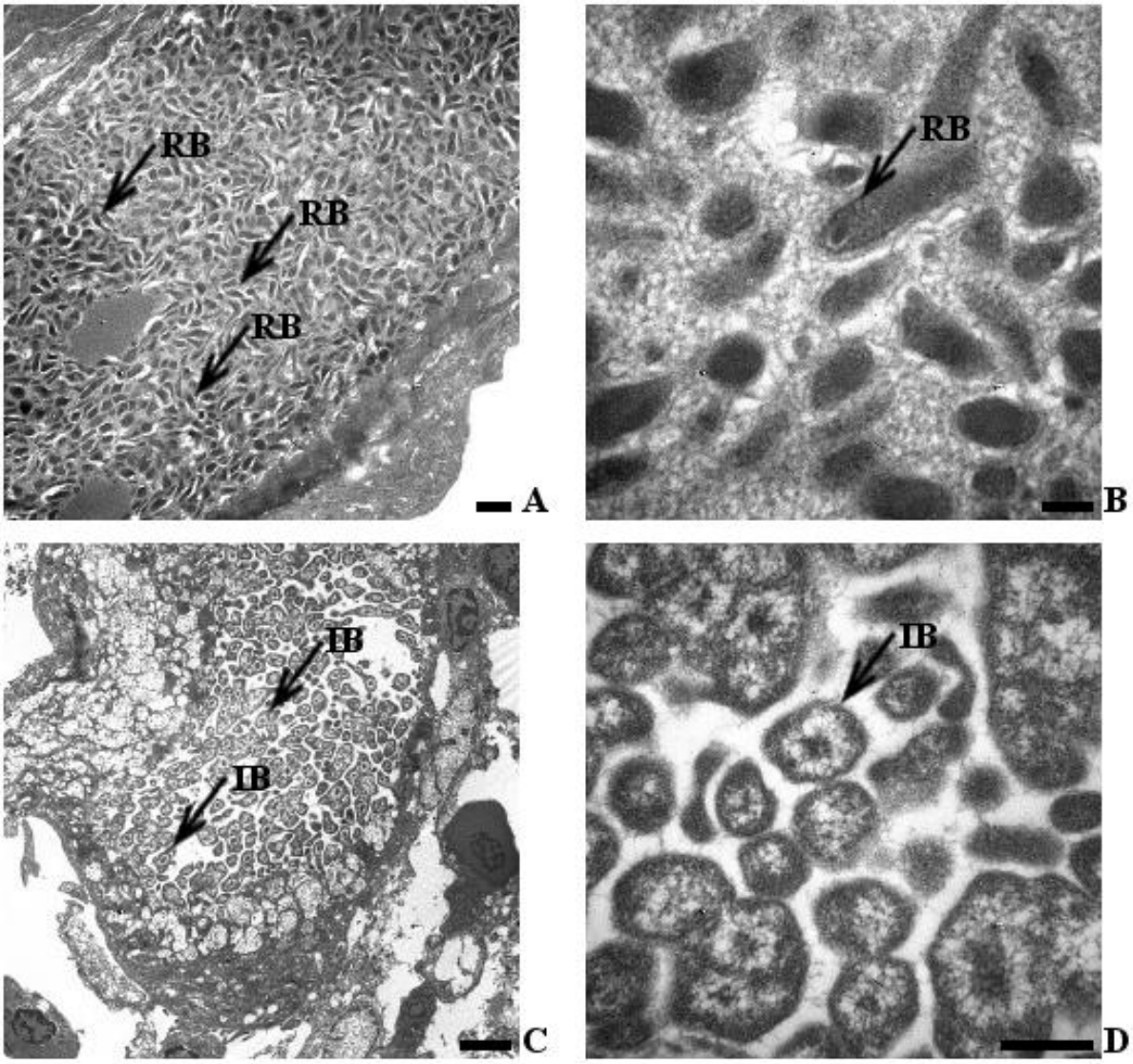

Figure 3.2: Transmission electron microscopy of *Chlamydia*-like epitheliocystis agent inclusions in gill epithelium of yellowtail kingfish (2002 cohort), *S. lalandi*. (A) Membrane-bound epitheliocystis inclusion body containing reticulate bodies (scale bar = 500 nm). (B) Higher magnification of reticulate bodies (RB) (scale bar = 100 nm). (C) Membrane-bound epitheliocystis inclusion body containing intermediate bodies (IB) (scale bar = 2 µm). (D) Higher magnification of intermediate bodies (scale bar = 500 nm). Note the absence of elementary bodies.

Table 3.1: Prevalence and intensity of infection of yellowtail kingfish, S. lalandi, with chlamydial DNA using both histology and PCR amplification.

Cohort	Histology				PCR prevalence (%)
	n	Prevalence (%)	Mean (± SE)[a]	Intensity	
2008	8	100	0.32 (0.062) A		100
2009	10	20	0.006 (0.004) B		80
2010	20	30	0.013 (0.005) B		80

[a] SE, standard error. Different letters denote significant differences ($P < 0.001$)

3.4.4 Molecular identification and phylogenetic analysis of novel *Chlamydia*-like organism

Initial order *Chlamydiales*-specific 16S rRNA PCR assay screening revealed that 100% of the 2008 (n = 8) and 2009 (n = 10) cohorts sampled and 80% of the 2010 cohort (16/20) screened PCR positive for chlamydial DNA. Sequences from five randomly selected samples per cohort were aligned and analysed and found to be identical within and between cohorts. Therefore, a single representative sample from each cohort was selected to identify the chlamydial species present by additional PCR amplification of extended and nearly complete 16S rRNA sequences. An expanded and nearly full-length sequence was amplified from a representative sample from each of the 2008 to 2010 cohorts (inclusive), and multiple-sequence alignment of the resulting 1,393-bp 16S rRNA sequence from all three samples indicated that they were 100% identical. The 16S rRNA PCR assay of the laser-dissected cysts was PCR positive for chlamydial DNA and was identical to the chlamydial 16S rRNA sequence obtained directly from the gill tissue.

BLAST-n analysis of the YTK epitheliocystis agent sequence against the NCBI database revealed the sequence to be novel, sharing only a distant 87% sequence similarity to the next closest 16S rRNA sequences from "*Candidatus* Piscichlamydia salmonis" (AY462243.1 and AY462244.1 (57); EU326495.1 (98)).

Alignment of the 16S rRNA data generated for the epitheliocystis agents isolated from YTK and the remainder of the *Chlamydiales* taxa and out groups examined (see Appendix S2) yielded 1,136 characters of analysis. The percentages of pairwise

identities observed between the family level taxa analysed over the 16S data set are shown in Table 3.2. Bayesian inference and maximum-likelihood analyses resulted in phylograms with identical topologies, which displayed all of the currently recognized and candidate families within the *Chlamydiales*, forming relatively well-supported clades (Figure 3.3). The sequence obtained from the novel epitheliocystis agent reported here was a sister taxon to the sequences available for "*Ca*. Piscichlamydia salmonis" and an uncultured *Chlamydiaceae*-like organism on GenBank (Figure 3.3). Phylogenetic comparisons between known epitheliocystis 16S rRNA signature sequences from GenBank also confirm the novel lineage of the novel CLO reported here (Figure 3.4). Furthermore, the chlamydial agent of epitheliocystis in YTK represents a novel family lineage in the order *Chlamydiales*.

Table 3.2: Estimates of evolutionary divergence between sequences.

Family	1	2	3	4	5	6	7	8
1) *Candidatus* Piscichlamydiaceae	-							
2) *Candidatus* Parilichlamydiaceae n. fam.	86-86.1%	-						
3) *Chlamydiaceae*	80.9-82.1%	80.9-81.6%	-					
4) *Candidatus* Rhabdochlamydiaceae	80.3-81.2%	81.1-81.7%	83-84.9%	-				
5) *Simkaniaceae*	80.8-82.3%	81.6-82%	83.6-85.2%	85.4-88.4%	-			
6) *Waddliaceae*	81.5-82.5%	80.8-81.7%	86-87.3%	84-86.2%	84.4-86.8%	-		
7) *Candidatus* Criblamydiaceae	82.1-82.6%	81.5-82%	86.1-87.9%	84.6-85.2%	84.3-85.8%	87-89.1%	-	
8) *Parachlamydiaceae*	80.8-82.2%	81.2-82.7%	85.2-87.5%	85-88.4%	85.5-88.6%	88.4-90.6%	87.2-91.3%	-

[a] The percentages of base similarities between families are shown. Sequences were trimmed, and there were a total of 1,136 nucleotide positions in the final data set. Evolutionary analyses were conducted in MEGA5 (49).

3.5 DISCUSSION

The novel YTK epitheliocystis bacterial agent from YTK has >80% sequence similarity to other members of the *Chlamydiales*, placing it within the order (71). At this stage, Koch's postulates have not been satisfied to definitively identify the sequenced agent as the cause of epitheliocystis, since this sequenced agent from YTK, and also other epitheliocystis CLO agents, has not been successfully cultured in vitro (57). However, the nearly full-length sequence of the CLO reported and confirmed here as identical in three different-year cohorts (sampled throughout the South Australian YTK farming zone) and the archival samples from 2002 from both the gill tissue and the cysts is strong evidence that it is a novel CLO bacterial agent causing epitheliocystis in YTK.

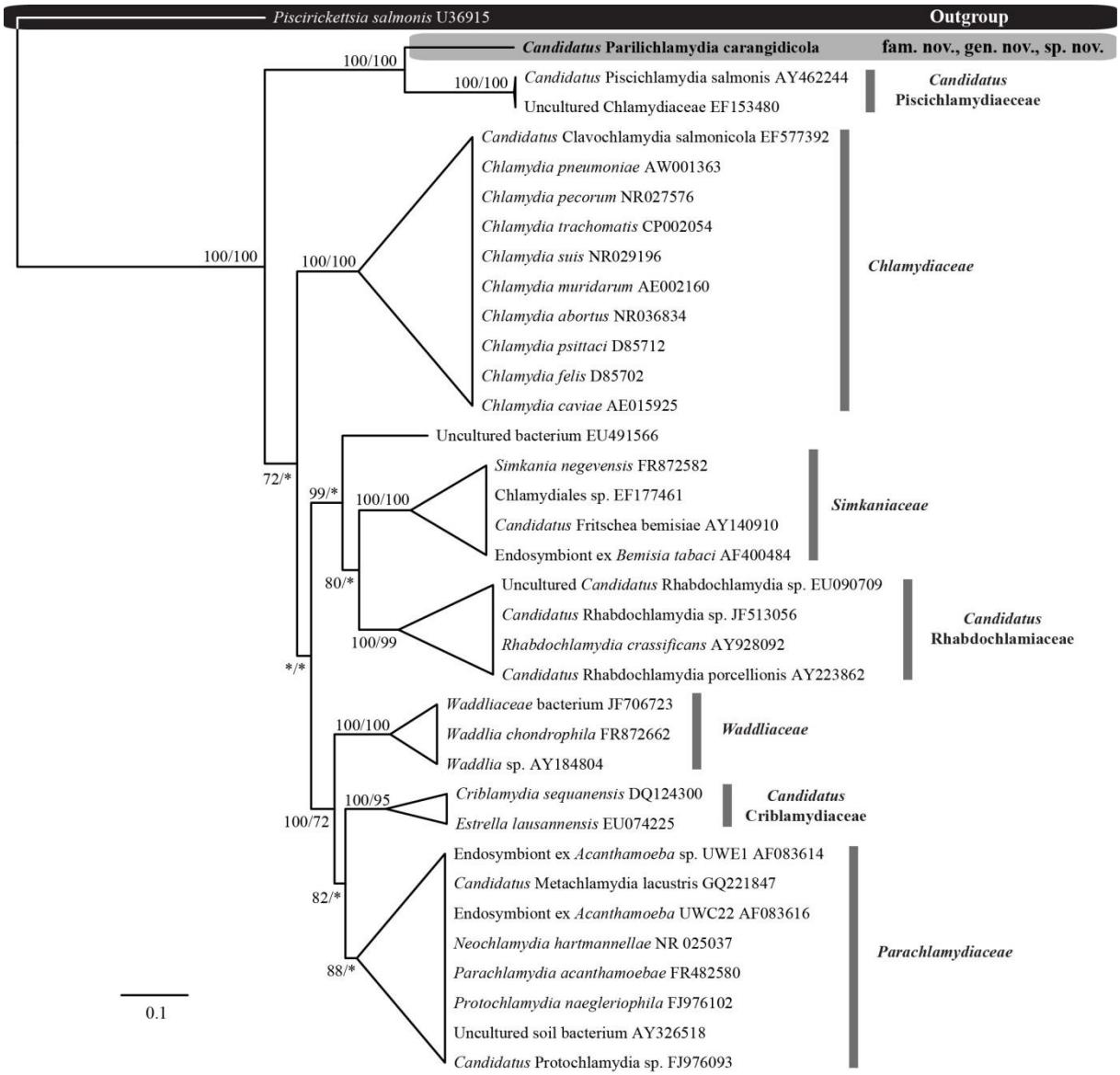

Figure 3.3: Relationships between the epitheliocystis agent detected in yellowtail kingfish gills and the remainder of the *Chlamydiales* taxa and out groups examined based on Bayesian inference maximum-likelihood analyses of the 16S rRNA data set. Posterior probability/bootstrap support values are given at the nodes, with values of less than 70% indicated by asterisks. Family clades have been collapsed.

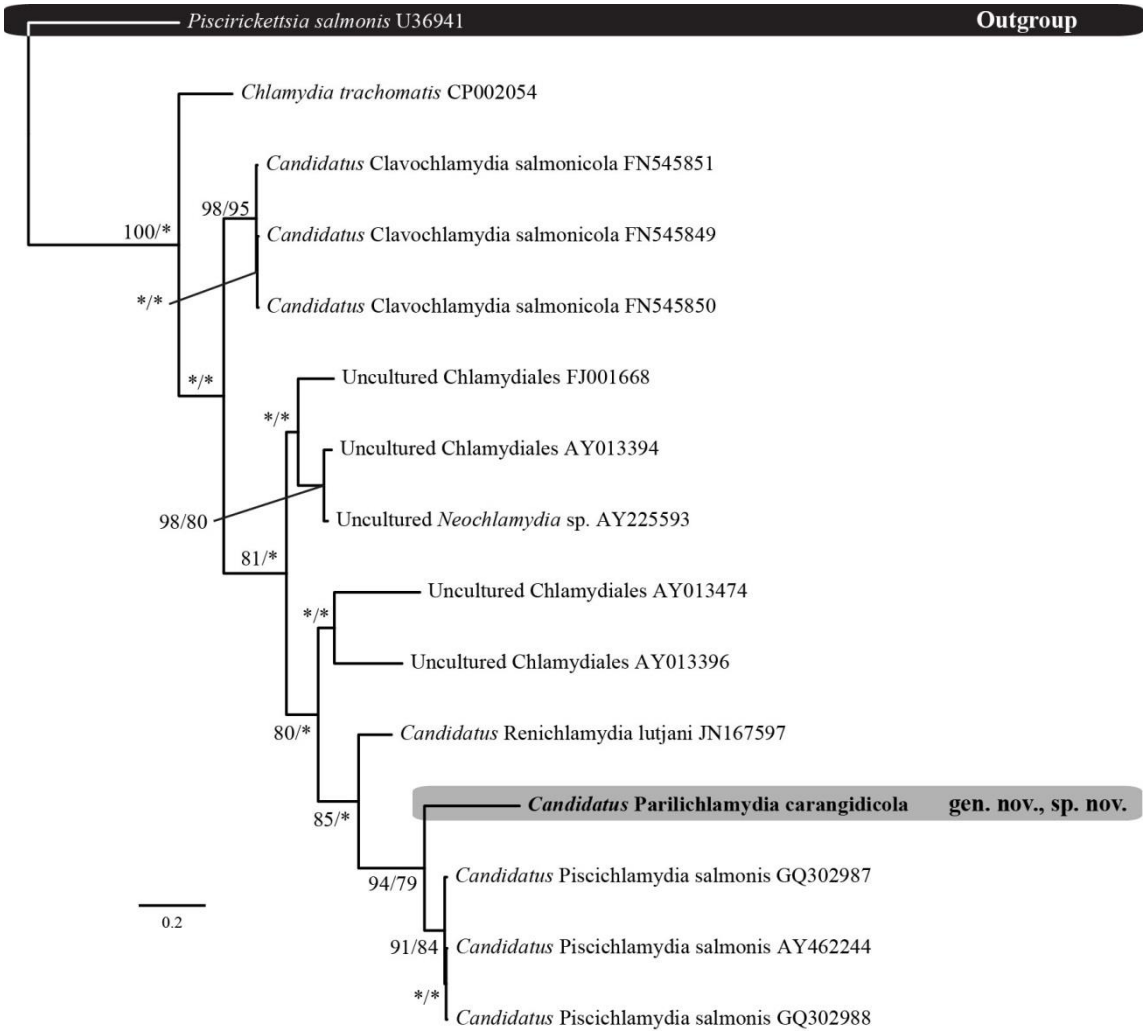

Figure 3.4: Relationship between the 298-bp signature sequence of the epitheliocystis agent detected in yellowtail kingfish gills and epitheliocystis agents detected in other fish species, *Chlamydia trachomatis* and *Piscirickettsia salmonis*. Bayesian inference maximum-likelihood analyses of the 298-bp signature sequence 16S rRNA data set were performed. Posterior probability/bootstrap support values are given at the nodes, with values of less than 70% indicated by asterisks.

Epitheliocystis has been noted as a recurrent problem in the culture of different yellowtail species, including *S. dumerili* (7) and *S. lalandi* from Ecuador (formally *Seriola mazatlana*) (17). Epitheliocystis infection in both *S. dumerili* and Ecuadorian *S. lalandi* resulted in a proliferative response, with enlarged cells packed with basophilic granules. However, lamellar fusion occurred only in Ecuadorian *S. lalandi* (17). Severe infection of epitheliocystis has been reported in cultured *S. lalandi* from South Australia, although the infection resulted in the presence and absence of a proliferative host response (10, 25). This difference in the host response may be attributed to the age of the fish. Older, larger *S. lalandi* fish appear to have less of a host response to epitheliocystis infections than juveniles. These reports of epitheliocystis in species of *Seriola* occurred prior to the widespread application of molecular techniques to the study of epitheliocystis, and molecular analyses have not been performed on the material to further characterize the epitheliocystis agent(s) involved. It will be of interest to determine if the epitheliocystis agent in these *Seriola* species, especially the Ecuadorian *S. lalandi*, is the same as the agent reported in this study.

This study also highlighted the discrepancies that can be found when using histopathology (20 to 100%) versus PCR (80 to 100%) for detection of epitheliocystis in fish. A similar discrepancy was also observed in a recent study of epitheliocystis in Atlantic salmon, where reverse transcription (RT)-PCR positivity ranged from 75 to 100% compared to 20 to 100% when using histopathology (27). Since histological methods continue to be the primary tool for detection of gill diseases in fish, these observations emphasise the fact that epitheliocystis, as a gill disease, is likely underdiagnosed in aquaculture settings, particularly in fish without any clinical signs of infection. While infection is not synonymous with disease, PCR is of course more sensitive for the detection of nonclinical and subclinical infections and possible infections that result in only a mild clinical disease. This detection is important, as it may provide the opportunity to institute changes in husbandry management to prevent a more serious condition within the population in the future.

While the cyst location at the base of the lamellae and the general morphology of the cysts are consistent with previous reports (36, 46, 47, 72, 74, 99, 100), the proliferative response of the YTK in this study was different than the response in Atlantic salmon. In

salmonids, epitheliocystis infection was associated only with mild lesions of the branchial epithelium, and any proliferative response was not associated with epitheliocystis (50, 86). The histological and electron microscopic features observed in YTK support the corresponding molecular result of a CLO being present within the epitheliocystis cysts. This agrees with previous reports for the molecular description of "*Ca*. Piscichlamydia salmonis" in Atlantic salmon (49, 57) and *Neochlamydia*-like bacteria in Arctic charr (35).

Although there have been two previous reports of nearly full-length 16S rRNA sequences, "*Ca*. Piscichlamydia salmonis" (57) and "*Ca*. Clavochlamydia salmonicola" (50), there is no single genus for *Chlamydia*-like bacteria associated with epitheliocystis (24). With the addition of this novel CLO, there are now three nearly full-length 16S rRNA sequences, all with 90% sequence similarity. According to accepted criteria, these bacteria belong to three separate family lineages within the *Chlamydiales* (58). Based on its novel 16S rRNA sequence, the percentage of sequence divergence from other *Chlamydiales* species, and the observed phylogenetic relationships of the bacteria to other taxa within the order, the name "*Candidatus* Parilichlamydia carangidicola" (gen. nov., sp. nov.) (Order *Chlamydiales*) is proposed to identify this *Chlamydia*-like epitheliocystis agent, whereas the name "*Candidatus* Parilichlamydiaceae" is proposed to identify the family lineage. Additional morphological and genetic data, including the sequencing of additional genes, will be required to formally characterize and classify this novel pathogen.

With this report, evidence continues to accrue concerning the taxonomic diversity of epitheliocystis agents from farmed and wild fish populations in marine and freshwater environments in both hemispheres. Further research will be required, not only to characterise the agents of the disease, but to evaluate the effects of risk factors, such as increased stocking densities (25), in response to increased demand for fish products, and environmental factors, such as water temperature (72, 101), that may promote and exacerbate epitheliocystis-related diseases.

3.5.1 Taxonomy

"*Candidatus* Parilichlamydia carangidicola" gen. nov., sp. nov. ("*Candidatus* Parilichlamydiaceae" fam. nov.), recovered from yellowtail kingfish (*Seriola lalandi*). *Parilichlamydiaceae* fam. nov.; Parili-, L. adj. parilis, equal or similar; *Chlamydiaceae*, N.L. fem. n., a bacterial family name. *Parilichlamydia* gen. nov.; Parili-, L. adj. parilis, equal or similar; *Chlamydia*, N.L. fem. n., a bacterial genus name. *Parilichlamydia carangidicola* sp. nov., *carangidicola* N.L. gen. sing. n., of Carangidae, the family to which the fish host belongs.

Obligate intracellular bacteria. Cells present tightly packed within a membrane-bound inclusion in vacuolated gill epithelial cells. These vacuoles contained elongated reticulate bodies and spherical intermediate bodies. Head and tail bodies were observed in the inclusions. There was a marked absence of elementary bodies. The reticulate bodies ranged in size from 83 by 211 nm to 100 by 361 nm, while the intermediate bodies ranged in size from 353 by 470 nm to 924 by 941 nm. The new family, genus, and species are distinguished from all other species of formally described and candidate *Chlamydiales* taxa based on a combination of the morphological and genetic differences (i.e., only 86 to 86.1% similarity to other *Chlamydiales* over the 16S rRNA data set examined) observed here. Fish are infected in the gills, with cellular hyperplasia and resulting vacuolation of cells and fusion of gill lamellae observed in histology.

3.6 Acknowledgements

We thank the following people for their assistance with this work: Nicole Kirchhoff and Ken Cain for assistance with sampling; Karine Cadoret for assistance and training in DNA extractions and histology; Brian Jones for laser dissection microscopy; and James Marsh, Eileen Roulis, and Martina Jelocnik for assisting with additional PCRs.

CHAPTER 4 MOLECULAR CHARACTERISATION OF "*CANDIDATUS* SIMILICHLAMYDIA LATRIDICOLA GEN. NOV., SP. NOV." (*CHLAMYDIALES*: *PARILICHLAMYDIACEAE*), A NOVEL *CHLAMYDIA*-LIKE EPITHELIOCYSTIS AGENT IN STRIPED TRUMPETER, *LATRIS LINEATA* (FORSTER)

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Keywords: *Latris lineata*, epitheliocystis, 16S rRNA, *Chlamydia*-like organism, striped trumpeter, wild, farmed

4.1 ABSTRACT

Histological analysis of gill samples taken from individuals of *Latris lineata* reared in aquaculture in Tasmania, Australia and those sampled from the wild revealed the presence of epitheliocystis-like basophilic inclusions. Subsequent morphological, *in situ* hybridisation, and molecular analyses were performed to confirm the presence of this disease and discovered a *Chlamydia*-like organism associated with this condition, and the criteria set by Fredericks and Relman's postulates were used to establish disease causation. Three distinct 16S rRNA genotypes were sequenced from 16 fish and phylogenetic analyses of the near full-length 16S rRNA sequences generated for this bacterial agent indicated that they were nearly identical novel members of the *Chlamydiales*. This new taxon formed a well-supported clade with '*Candidatus* Parilichlamydia carangidicola' from yellowtail kingfish (*Seriola lalandi*). On the basis of

sequence divergence over the 16S rRNA region relative to all other members of the order *Chlamydiales,* a new genus and species are proposed here for the *Chlamydia*-like bacteria from *L. lineata*, i.e., "*Ca.* Similichlamydia latridicola" gen. nov., sp. nov.

4.2 INTRODUCTION

The striped trumpeter, *Latris lineata* (Forster 1801), is distributed in southern hemisphere waters from the Walters Shoals (43°50'E) and Amsterdam Island (77°33'E) in the Indian Ocean through the southern waters of Australia and then to Chatham Island (176°29'W) in the Pacific Ocean (http://www.fishbase.org). The overexploitation of this species throughout its range has led to a significant decrease in the wild population, with the total commercial catch decreasing in the last 20 years by almost 100 tonnes to 12.8 tonnes in 2009/10 (19, 20). Because of the marked decline in wild stocks, the culture of *L. lineata* has been in development at the Tasmania Aquaculture and Fisheries Institute, Hobart, Tasmania, for 16 years. *Latris lineata* is considered to be a suitable aquaculture candidate, however, issues associated with its complex and lengthy 9-month postlarval stage have been difficult to overcome (19). The life cycle of *L. lineata* has now been successfully closed, and established protocols exist for its reproduction and larval rearing (21). Despite this, a number of health issues were observed between 1994 and 2010 during the development of *L. lineata* for commercial aquaculture. Examples included abnormal swimming behaviour, anorexia, swim bladder hyperinflation, skin lesions, and inflammation and swelling of gills in cultured juveniles due to infections from *Kudoa neurophila* and chondracanthid copepods (102, 103). In determining the causes of these health issues, epitheliocystis was also described in these fish (73). The latter disease is a condition of the skin and gills and is generally associated with infections by *Chlamydia*-like organisms (CLOs) (25, 27, 50, 70). These CLOs are Gram-negative, intracellular bacteria that may cause cyst-like lesions in the gill lamellae (24, 27, 86). The lesions may lead to epithelial hyperplasia and inflammation of the infected tissues, increased mucus production, and respiratory distress, sometimes ending in death (24, 35, 46, 47). Most reported losses in aquaculture attributed to epitheliocystis occur during the larval or juvenile culture stage (54).

Little is known about the epidemiology and pathogenesis of epitheliocystis agents. In an effort to understand this, researchers have turned from traditional microbiology

methods to molecular techniques in an attempt to understand this condition. This has led to a move towards fulfilling the Fredericks and Relman's molecular postulates instead of Koch's postulates (67). As a result, the primary method now used to describe and characterise unknown epitheliocystis agents taxonomically include phylogenetic analysis of DNA sequence data in combination with morphological descriptions. Following this trend, "*Candidatus* Parilichlamydia carangidicola" was recently reported from yellowtail kingfish in Australia by using molecular techniques and transmission electron microscopy as primary evidence (70).

The objective of this study was to identify and characterise the agent causing epitheliocystis in *L. lineata*, both in cultured individuals and fish obtained from the wild. Histological examination of epitheliocystis infections in the gill were confirmed by PCR of the 16S rRNA gene and *in situ* hybridisation. Following this, Bayesian inference and maximum likelihood phylogenetic analyses were performed using 16S rRNA sequences to explore the relationships of the striped trumpeter epitheliocystis agent with other epitheliocystis agents in fish, and with other members of the order *Chlamydiales*.

4.3 Materials & Methods

4.3.1 Ethics statement

Sampling of animals for this study was approved by the University of Tasmania Board of Animal Ethics, project number AEC0009926.

4.3.2 Sample collection

Latris lineata were reared in 20,000 L recirculated and flowthrough tanks at the Tasmanian Aquaculture and Fisheries Institute (TAFI), Hobart, Tasmania. Most fish were held in temperature- and light-controlled flowthrough recirculation tanks with 50% fresh sea water (sand and bag filtered [50 μm]) exchange three times a week. Some fish were in tanks on flowthrough seawater supply with only coarse particle filters. A total of 87 cultured fish were sampled at two time points, July 2010 ($n = 8$) and November 2010 ($n = 79$). The November 2010 samples were broodstock originally captured from south-eastern and north-eastern Tasmania and the F1 generation bred in captivity. All broodstock fish had been in captivity for at least 5 years and were not separated by origin. Fish were euthanased with 0.04% 2-phenoxyethanol and then

weight and length measurements were taken. In addition to the cultured striped trumpeter, wild fish ($n = 6$) were sampled from waters of south-western Tasmania (43°32'48" S, 145°56'27" E). For all fish, the second gill arch on the sinistral side was sampled, with the first subsection fixed in seawater Davidson's fixative (cultured) or 10% neutral buffered formalin (wild) for histology and the second subsection frozen at -80°C (cultured) or placed in RNAlater (wild) for DNA extractions.

4.3.3 Histopathology

Seawater Davidson's-fixed and formalin-fixed gills were routinely processed for histology. The gills were sectioned at 5 µm and stained with haematoxylin and eosin. The sections were examined by light microscopy to identify epitheliocystis inclusions and associated lesions (70).

4.3.4 DNA extraction, 16S rRNA amplification and sequencing

DNA was extracted from samples using the commercially available Epicentre MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's instructions and optimised as described previously (70). Extracted DNA from the July 2010 ($n = 8$), November 2010 ($n = 5$), and wild ($n = 3$) cohorts was screened by a conventional *Chlamydiales*-specific PCR assay targeting a 298 bp "signature sequence" region of the 16S rRNA gene with the primers 16SIGF and 16SIGR, as described previously (28, 58, 70). Expanded 800 bp (with primers 16SIGF and 806R) and nearly full-length (with primers 16SIGF and 16SB1) 16S rRNA sequences from selected representative samples were completed. PCR amplification, cycling conditions, purification and sequencing for these assays were as previously described (70).

4.3.5 *In situ* hybridisation

Detection of the *Chlamydia*-like organism within the epitheliocystis cysts by ISH in 5 µm serial sections was conducted with the *Chlamydiales*-specific antisense (digoxigenin [DIG]-ATG TG[T/C] TAC TAA CCC TTC CGC CAC TA-DIG) and sense (DIG-ATC CTA CGC TAC TAA GTC TCT CAT CA-DIG) DIG-labeled oligonucleotide probes as described previously (24, 104), with some modifications. Briefly, the sections were dewaxed, washed with phosphate-buffered saline (PBS) for 3 min, and digested for 30 min at 37°C

in 5 mg/L proteinase K (Sigma) in 0.1 mol/L Tris-HCl (pH 7.6). The sections were washed twice with 0.2% glycine in PBS for 5 min, once with 0.01% triton X-100 in PBS for 10 min and twice with PBS for 5 min; dehydrated in 95% and 100% ethanol for 3 min each, and then dried. The hybridization mixture, containing 42% deionized formamide, 9.4% Dextran sulfate, 5.8X saline-sodium citrate (SSC) buffer (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 4.7X Denhardt's solution, 94 mg/L denatured salmon sperm DNA, and the probe(s) at a final concentration of 10.2 pmol/mL, was heated for 10 min at 100°C, cooled on ice, and added to the sections. The sections were incubated at 95°C for 5 min prior to overnight incubation at 55°C in a humid chamber. The sections were washed twice with 2X SSC for 15 min, once with 1X SSC for 5 min, once with 0.5X SSC for 5 min and twice with Tris-HCl (pH 7.6) for 10 min. Blocking buffer (containing 0.1% triton X-100, 2% normal sheep serum and PBS) was added, and the sections were incubated for 30 min at room temperature. The hybridized probes were visualized by the addition of alkaline phosphatase-labeled anti-DIG Fab fragments (α-DIG-AP), incubation in a humid chamber for 2 h, washed twice with Tris-HCl (pH 9.5) for 10 min, chromogen (5-bromo-4chloro-3-indolyl-β-D-galactopyranoside –Nitro Blue Tetrazolium [BCIP/NBT]) addition, and incubation in a humid chamber for 75 min. The sections were then counterstained with Nuclear Fast Red (Sigma) and mounted with Vectamount.

4.3.6 Molecular phylogenetic analysis

The partial 16S rRNA regions sequenced here and data from additional *Chlamydiales* species and out-group taxa obtained from GenBank were aligned as previously described (70). The software jModelTest version 0.1.1 (105, 106) estimated TVM+I+G as the best nucleotide substitution model for this dataset. Bayesian inference and maximum likelihood analyses of the 16S rRNA dataset was performed using MrBayes version 3.1.2 (95) and RAxML algorithm (97), respectively, run on the CIPRES portal (96) to explore relationships among these taxa under conditions set as previously described (70).

4.3.7 Quantification

The prevalence (expressed as a percentage) and intensity (intensity = cysts per section/filaments per section) of epitheliocystis infection were calculated for each fish

after visual inspection of haematoxylin-eosin-stained gill sections. The prevalence of CLOs detected by PCR was also calculated (expressed as a percentage). Statistical analyses were conducted with the IBM SPSS Statistics package, version 20.0.0.1 (2011). Levene's test was performed to assure the assumption of homogeneity of variances. One-way analysis of variance (ANOVA) was performed to test the fish length and infection intensity against the sampling points.

4.3.8 Nucleotide sequence accession number

The three 16S rRNA gene sequences of the *L. lineata* epitheliocystis agents determined in this study are available in the GenBank database under the accession numbers JQ687061, KC686678 and KC686679.

4.4 RESULTS

4.4.1 Histopathology and prevalence of novel striped trumpeter *Chlamydia*-like organism

The mean fish length, weight and the prevalence (%) of the infectious agent at each sampling point are summarised in Table 4.1; the origins of the fish are also shown. There was no significant difference in fish length between the sampling points ($F = 3.802$, $df = 1$, $p = 0.055$). Epitheliocystis, as determined by histological examination, was present in fish sampled at all sampling points. The prevalence ranged from 50% in fish of wild origin (August 2011) to 100% in broodstock held in captivity (November 2010; Table 4.1). There was no significant difference in the intensity of epitheliocystis between the sampling points ($F = 1.750$, $df = 2$, $p = 0.180$). Infection intensity ranged from 0.02 to 3.37 cysts/filament in the November 2010 fish, from 0.07 to 2.43 cysts/filament in the July 2010 fish, and from 0.04 to 0.07 cysts/filament in the wild fish sampled in August 2011. Membrane-enclosed granulated basophilic cysts were present along the entire length of the filaments in affected fish from all three sampling points (Figure 4.1 B). In terms of the host response to these cysts, hyperplasia of epithelial cells could be observed in some, but not all, of the cultured fish from both sampling points while no responses were observed in infected wild fish (Figure 4.1 A and 1B).

Table 4.1: Mean length (mm), mean weight (kg), prevalence and intensity of epitheliocystis infections in striped trumpeter sampled during July 2010, November 2010 and August 2011. The number of fish that were PCR positive and their corresponding genotype are shown.

Parameter	July 2010 F1 Generation	November 2010 Broodstock	August 2011 Wild
No. of samples	8	79	6
Mean length, mm (SE)	ND[a]	539 mm (9.2)[b]	605 mm (19.0)[b]
Mean weight, kg (SE)	ND	2.80 kg (0.13)	ND
Prevalence, % (*n* tested)	100% (8/8)	75.9% (60/79)	50% (3/6)
Intensity (cysts/filaments) (SE)	0.55 (0.28)[b]	0.46 (0.16)[b]	0.03 (0.01)[b]
PCR Positive (no. tested)	100% (8)	100% (5)	100% (3)
Genotype	A/B	C/A	B
Origin	TAFI[c], Hobart	TAFI, Hobart[d]	SW[e] Tasmania[f]

[a] ND, no data; [b] Statistically significantly different; [c] TAFI, Tasmanian Aquaculture & Fisheries Institute; [d] Broodstock fish were held in captivity for >5 years and were originally from waters around Flinders Island (northeastern Tasmania) and Tasman Island (southeastern Tasmania); [e] SW, southwestern; [f] Fish were caught at 43°55'480"S, 145°56'272"E.

4.4.2 Molecular identification and phylogenetic analysis of novel *Chlamydia*-like organism

Preliminary screening with a *Chlamydiales*-specific 16S rRNA PCR assay revealed that 100% ($n = 16$) of the striped trumpeter samples screened PCR positive for chlamydial DNA (July 2010, n = 8; November 2010, $n = 5$; Wild 2011, n = 3). Pairwise alignments of sequences revealed three distinct genotypes with >99% nucleotide sequence similarity to each other. Six single nucleotide polymorphisms (SNPs) were present between the three nearly full-length genotype sequences (1,396 bp). The SNPs were consistently found at the same positions within the gene whether using the 16SIGF/16SIGR, 16SIGF/806R or the 16SIGF/16SBI primer pair and were sequenced in multiple samples. In addition, these SNPs were positioned within the variable region of the signature sequence (bp 40 – 337 of the 16S rRNA gene) as outlined by Everett (58). Genotype A and B were found multiple times and from multiple origins. Genotype A was found from both July 2010 and November 2010 samples (Table 4.1). The fish sampled in July 2010 were also positive for genotype B, which was sequenced from the wild fish sampled in August 2011. Finally, genotype C was sequenced from fish sampled in November 2010 only (Table 4.1).

BLAST-n analysis of the consensus *L. lineata* CLO sequences against the NCBI database revealed these sequences to be novel, sharing 93.7 – 94.0% sequence similarity to the next closest 16S rRNA sequence, "*Ca.* Parilichlamydia carangidicola", a recently reported novel *Chlamydia*-like epitheliocystis agent in yellowtail kingfish, *Seriola lalandi* (70). The next closest sequence (88% sequence similarity) identified belonged to "*Ca.* Piscichlamydia salmonis" from the Atlantic salmon (accession no. AY462244, (57); EU326495, (98)) and the Arctic charr (GQ302987, (49)).

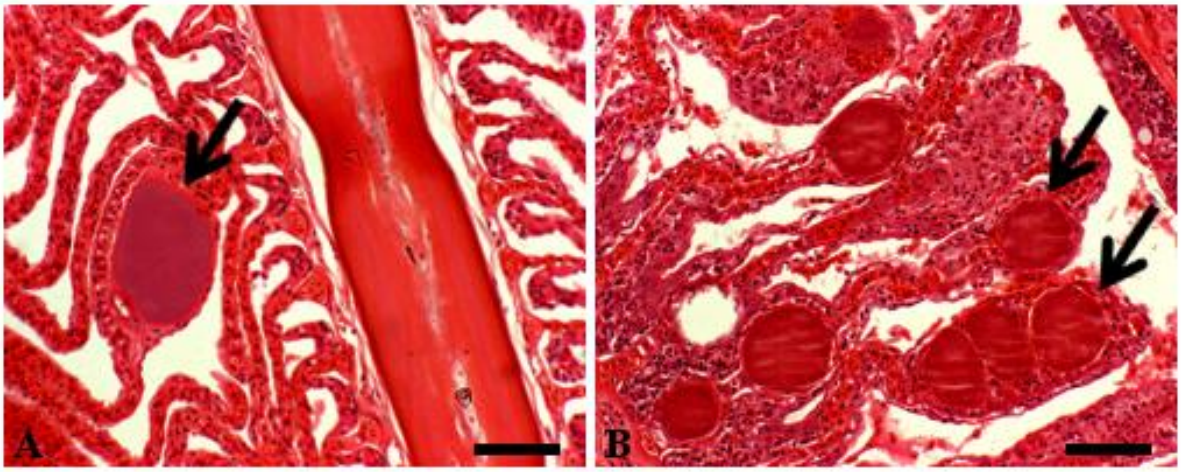

Figure 4.1: Epitheliocystis in striped trumpeter (*Latris lineata*) gills stained with haematoxylin and eosin; (A) a singular membrane-enclosed basophilic granular cyst with no host response (scale bar = 50 µm); (B) multiple membrane-enclosed basophilic granular cysts along individual lamellae with a hyperplasic epithelia host response (scale bar = 50 µm).

Alignment of the 16S rRNA data generated for the epitheliocystis agent isolated from striped trumpeter (*Latris lineata*) and the remainder of the *Chlamydiales* taxa and outgroups examined here yielded 1,127 characters of analysis. Bayesian inference and maximum likelihood analyses resulted in phylograms with markedly similar topologies, with all of the currently recognised and candidate families within the order *Chlamydiales* forming relatively well-supported clades (Figure 4.2). Alignment and analysis of the shorter signature sequence region of the 16S rRNA showed that the epitheliocystis agent reported here from *L. lineata* grouped together in a strongly

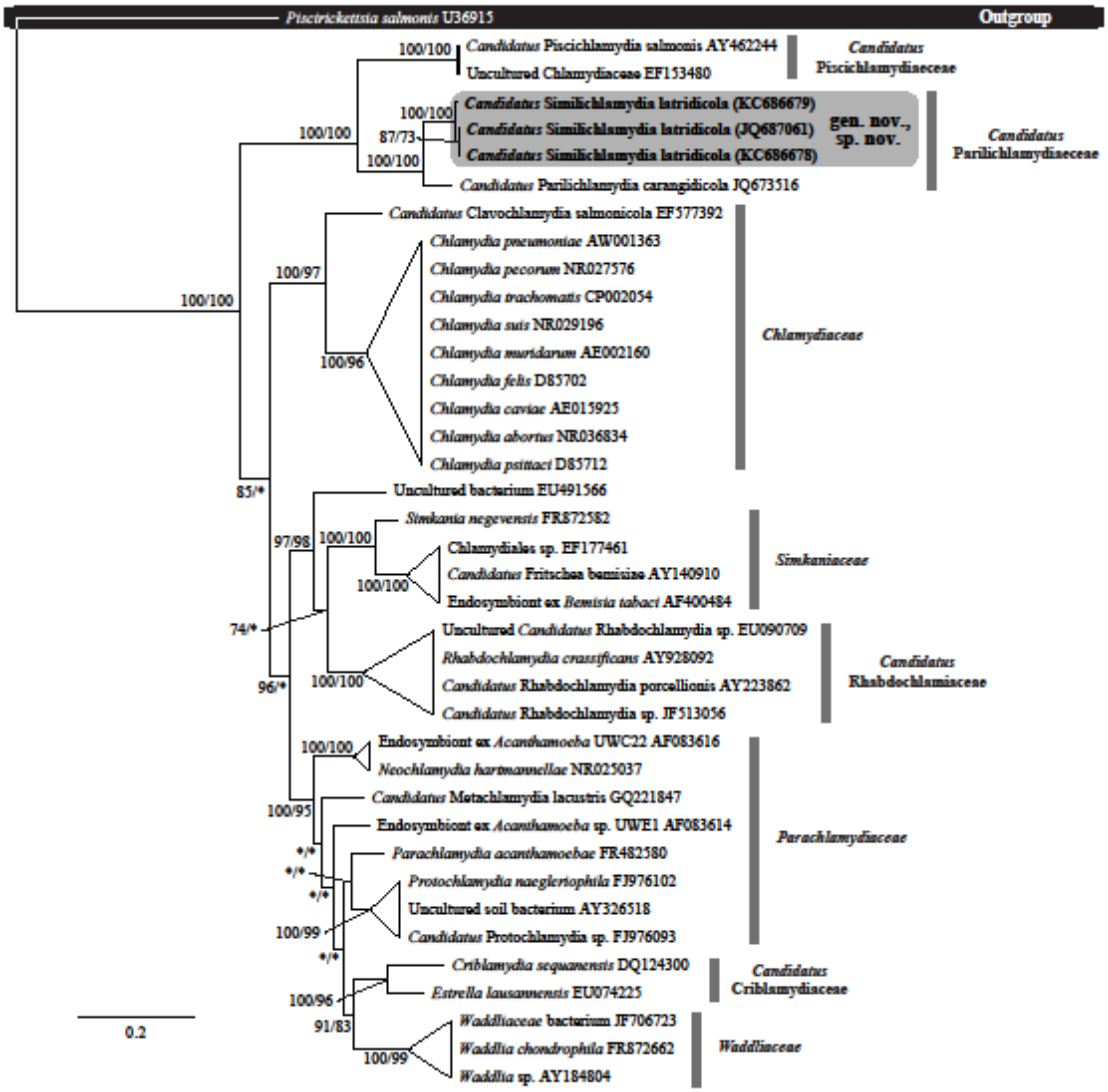

Figure 4.2: Relationships between the epitheliocystis agents isolated from the striped trumpeter (*Latris lineata*) and the remainder of the *Chlamydiales* taxa and outgroups examined here based on Bayesian inference and maximum likelihood analyses of the 16S rRNA dataset. Posterior probability and bootstrap support values (respectively) are given at the nodes, with values less than 70% indicated by an asterisk. Clades of representative genera have been collapsed.

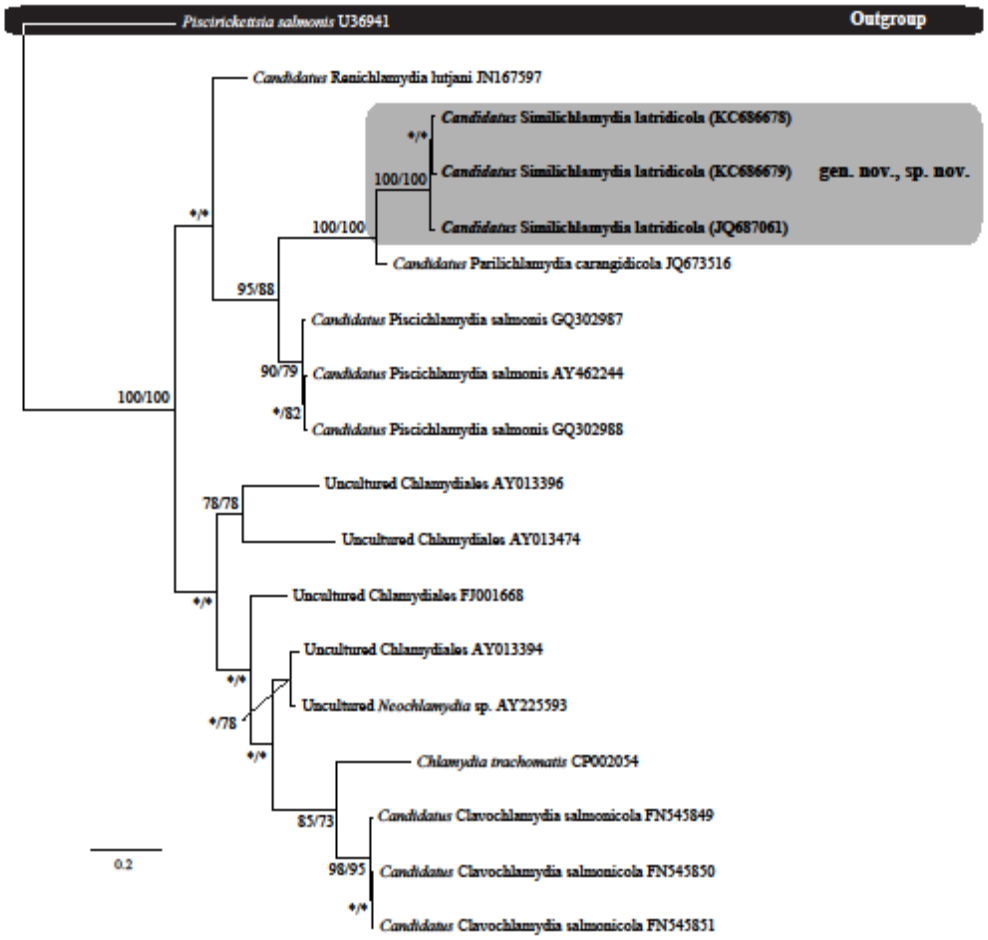

Figure 4.3: Relationships between the 298 bp (16S rRNA) signature sequence of the epitheliocystis agent detected in striped trumpeter (*Latris lineata*) gills and epitheliocystis agents detected in other fish species, *Chlamydia trachomatis* and *Piscirickettsia salmonis* based on Bayesian inference and maximum likelihood analyses. Posterior probability and bootstrap support values (respectively) are given at the nodes, with values less than 70% indicated by an asterisk.

supported clade that was sister to the sequences available for "*Candidatus* Parilichlamydia carangidicola", multiple sequences available for "*Candidatus* Piscichlamydia salmonis" and the sequence available for "*Candidatus* Renichlamydia lutjani" in GenBank, which have all been reported from teleosts (Figure 4.3).

4.4.3 *In situ* hybridisation

The presence of *Chlamydia*-like sequences detected in epitheliocystis positive striped trumpeter gill samples by PCR was confirmed by ISH using the *Chlamydiales*-specific probes. Epitheliocystis cysts reacted strongly to the antisense probe, with cysts staining a dark purple/black colour. Epitheliocystis cysts that were incubated with the sense probe showed no reactivity (Figure 4.4 A, B).

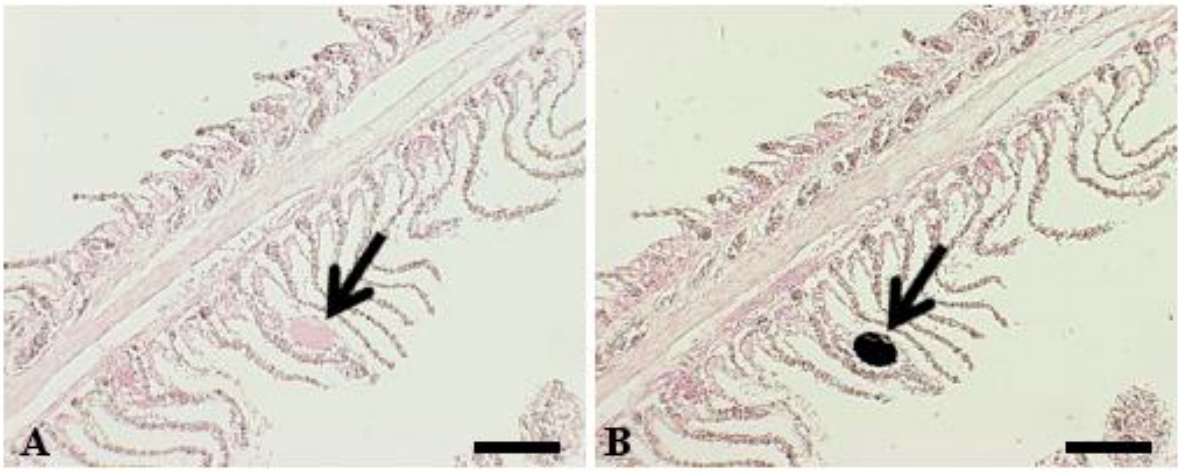

Figure 4.4: Detection of *Chlamydiales* bacteria in epitheliocystis infected gills of striped trumpeter (*Latris lineata*) by *in situ* hybridisation; (A) reaction of epitheliocystis gill with the sense ISH probe, note the absence of a reaction with the cyst (scale bar = 100 µm); (B) reaction of epitheliocystis cysts to the antisense ISH probe, note the purple/black colouration of the cyst indicating a positive reaction (scale bar = 100 µm).

4.5 DISCUSSION

Epitheliocystis is now known to affect over 80 different species of fish (25, 70), and intriguingly, from all of these reports, it appears that the host responses considered to be severe or as having a hyperinfection are all from farmed fish (26, 35, 36, 47, 49, 54,

100, 107, 108). Molecular diagnostics confirmed that this CLO, from both cultured and wild sources, is a member of the order *Chlamydiales*, based on the >80% sequence similarity to other species in the order (58). The nearly identical, nearly full-length 16S rRNA sequences of the CLO obtained over the three sampling periods and from both wild and cultured fish are strong evidence that it is the agent causing epitheliocystis in striped trumpeter fish. The detection of *Chlamydiales*-specific 16S rRNA gene in striped trumpeter epitheliocystis gill inclusions by ISH with DIG-labelled probes provides further evidence that the cysts identified by light microscopy are the source of the amplified novel 16S rRNA gene sequence(s).

The prevalence of epitheliocystis and the response of the striped trumpeter to infection reported in this study are in line with that previously reported for this species (73). The response seen in this study, however, was not as severe as that in previous reports of hyperinfection in the largemouth bass, *Micropterus salmoides*, and the Atlantic salmon, *Salmo salar* (27, 100). Epithelial hyperplasia, epithelial lifting along the lamellae and lamellar fusion in the striped trumpeter were observed, although the filling of the interlamellae spaces was not as severe as that in previous reports. This differs from the nearly complete filling of the interlamellae spaces reported in both the largemouth bass (100) and the Arctic charr (35, 49), which resulted in a severely compromised respiratory system.

Striped trumpeter fish were recently reported to be affected by epitheliocystis (73). While that was the first report of epitheliocystis from the family Latridae, the condition has been reported in other species in the superfamily Cirrhitoidea, including the rock cale, *Crinodus lophodon* and the red morwong, *Cheilodactylus fuscus* (74). Like the striped trumpeter, both of these species originate from Australian waters. In these other species, benign cysts with little or no host response were observed in the gills, which matched that of the wild fish and some of the cultured striped trumpeter from this study (74). Unfortunately, no molecular data on the identity of the epitheliocystis agent from infections in these species are available comparison, as the molecular techniques used here were not common practice at that time.

Questions remain about the origin of this infection and the potential impact that this novel epitheliocystis agent may have on the health and productivity of striped trumpeter. In the cultured environment, the water source and treatment, and the tank environmental conditions (temperature, salinity, dissolved oxygen and nitrates) are controlled and monitored daily by staff. However, since infections were found in broodstock from both south-western and north-western Tasmanian waters, and in fish sampled from the wild, it is reasonable to conclude that epitheliocystis occurs naturally in the environment and was introduced to the culture systems either with the fish or with sea water supply. Although the bacterial sequences obtained from the different sources were of three distinct genotypes, they are extremely closely related. The development of *in vitro* methods for culturing these bacteria is needed to help answer questions on how variable these organisms naturally are, how these organisms are transmitted within a population, and what environmental factors, if any, may lead to hyperinfection.

Because of the current inability to culture *Chlamydia*-like bacteria *in vitro*, an alternative to Koch's postulates must be used. The molecular postulates of Fredericks and Relman were therefore used in this study (67). The nearly identical sequences identified here from both wild and cultured sources were present in all cases of disease observed in histology. The nature of the CLOs detected here is consistent with the known biological characteristics of *Chlamydia;* that is, they are intracellular bacteria requiring a host cell to replicate. The order specificity of the sequences were detected within the epitheliocystis cysts through ISH; and all these results are repeatable. These results are in line with the molecular postulates of disease causation (67) and provide strong evidence that the epitheliocystis agent of striped trumpeter is of *Chlamydiales* origin. On the basis of its novel 16S rRNA signature sequence, the sequence divergence from other *Chlamydiales* species and the observed phylogenetic relationships of this bacterium to other taxa within the order according to their classification (58), we propose the name "*Candidatus* Similichlamydia latridicola" (gen. nov., sp. nov.) for the *Chlamydia*-like epitheliocystis agent infecting striped trumpeter.

4.5.1 Taxonomy

"*Candidatus* Similchlamydia latridicola" gen. nov., sp. nov., recovered from striped trumpeter (*L. lineata*). *Similichlamydia* gen. nov.; Si.mi.li.chla.my'di.a. L. adj. similis, resembling; N.L. fem. n. *Chlamydia*, a bacterial genus name; N.L. fem. n. Similichlamydia, resembling *Chlamydia*. latridicola sp. nov.; la.tri.di.'co.la. N.L. n. Latris -idis, a zoological genus name; L. suff. -cola (from L. n. incola), inhabitant, dweller; N.L. n. latridicola, Latris-dweller, isolated from striped trumpeter (*Latris lineata*).

Obligate intracellular bacteria infecting fish gills. Membrane-bound inclusions present as granular and tightly packed, staining basophilic under haematoxylin and eosin. Inclusions are found along the gill filament at the base, middle and tip of the lamellae and incite a host response of cellular hyperplasia. Inclusions react with ISH 16S rRNA probe and stain purple/black. The new genus and species 16S rRNA sequence is 6.0 – 6.3% different from the 16S rRNA of "*Candidatus* Parilichlamydiaceae" placing it within this family, but not a member of the genus "*Candidatus* Parilichlamydia" according to the classification scheme of Everett (58).

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CHAPTER 5 "*CANDIDATUS* SIMILICHLAMYDIA LATICOLA", A NOVEL *CHLAMYDIA*-LIKE AGENT OF EPITHELIOCYSTIS IN SEVEN CONSECUTIVE COHORTS OF FARMED AUSTRALIAN BARRAMUNDI, *LATES CALCARIFER* (BLOCH)

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Keywords: barramundi, *Lates calcarifer*, epitheliocystis, 16S rRNA, *Chlamydia*-like organism, barramundi

5.1 ABSTRACT

Six consecutively hatched cohorts and one cohort of pre-hatch eggs of farmed barramundi (*Lates calcarifer*) from South Australia were examined for *Chlamydia*-like organisms associated with epitheliocystis. To identify and characterise the bacteria, 59 gill samples and three pre-hatch egg samples were processed for histology, in situ hybridisation and 16S rRNA amplification, sequencing and comprehensive phylogenetic analysis. Cases of epitheliocystis were observed microscopically and characterised by membrane-enclosed basophilic cysts filled with a granular material that caused hypertrophy of the epithelial cells. *In situ* hybridisation with a *Chlamydiales*-specific probe lead to specific labelling of the epitheliocystis inclusions within the gill epithelium. Two distinct but closely related 16S rRNA chlamydial sequences were amplified from gill DNA across the seven cohorts, including from pre-hatch eggs. These genotype sequences were found to be novel, sharing 97.1 – 97.5% similarity to the next closest 16S rRNA sequence, *Ca*. Similichlamydia latridicola, from Australian striped trumpeter. Comprehensive phylogenetic analysis of these genotype sequences against

representative members of the *Chlamydiales* order and against other epitheliocystis agents revealed these *Chlamydia*-like organisms to be novel and taxonomically placed them within the recently proposed genus *Ca*. Similichlamydia. Following Fredricks and Relman's molecular postulates and based on these observations, we propose the epitheliocystis agents of barramundi to be known as "*Candidatus* Similichlamydia laticola" (sp. nov.).

5.2 INTRODUCTION

Aquaculture is the fastest growing primary industry in Australia and, in 2011, a total of 75,188 tonnes of fish were produced with a value of almost one billion dollars to the Australian economy (2). Established species such as the barramundi (*Lates calcarifer*) (Bloch, 1790), which was first developed in the mid-1980s and is now farmed in every state of Australia except for Tasmania, are strong contributors to this output. In 2011, barramundi aquaculture accounted for over 4,000 tonnes of product annually with a value of 36 million dollars (2). Although barramundi aquaculture is well-established, an increased incidence of viral, bacterial and parasitic diseases occurs within the higher densities of farmed populations (109).

Epitheliocystis is a condition of the gills and skin of finfish and is caused by intracellular Gram-negative bacteria. It occurs in both wild and farmed fish populations and is currently known to affect over 80 different species of marine and freshwater fish (70, 72), including barramundi (24, 71). Epitheliocystis is usually associated with *Chlamydia*-like organisms (CLOs) (50, 70, 72, 86), which can cause epithelial hyperplasia, hypertrophy and inflammation of the infected tissue (24, 46). The identification of *Chlamydia*-like bacteria in association with epitheliocystis is increasing, with several new reports of new *Candidatus* species being described, namely *Ca.* Piscichlamydia cyprinis from grass carp (*Ctenopharyngodon idella*) (51), *Ca.* Parilichlamydia carangidicola from yellowtail kingfish (*Seriola lalandi*) (70) and *Ca.* Similichlamydia latridicola from striped trumpeter (*Latris lineata*) (110).

Epitheliocystis was previously described in the barramundi and the aetiological agent was thought to be a unique *Chlamydiae*, however, identification of this agent was limited to the amplification and sequencing of short 16S rRNA sequences from fixed tissue

specimens (24, 71). The prevalence and factors associated with infections by this or other aetiological agents of epitheliocystis in barramundi are otherwise unknown.

In the current study, we have performed a cross-sectional survey of six consecutively hatched cohorts of barramundi, plus pre-hatch eggs, for the presence and effects of epitheliocystis infections in a barramundi aquaculture facility. Fredricks and Relman's molecular postulates were used with detailed histopathology, *in situ* hybridisation (ISH), near-full length 16S rRNA gene sequences and complete phylogenetic analyses as evidence for disease causation (67).

5.3 MATERIALS & METHODS

5.3.1 Ethics statement

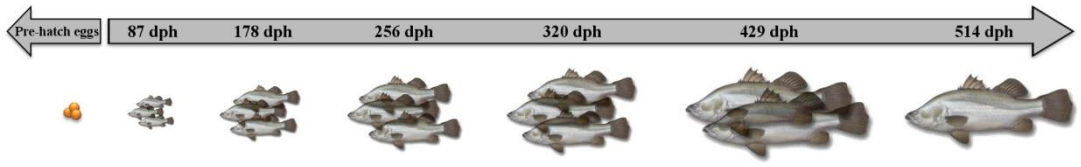
The samples were collected as a part of routine farm health monitoring and provided to the researchers as fixed samples. As such they were exempt from Ethics approvals (confirmed by University of Tasmania Ethics Committee).

5.3.2 Sample collection

Barramundi were spawned and reared at a commercial aquaculture facility in South Australia. Fish less than 100 mm in total length were raised in 1 m^3 cages or 2 m^3 flow-through tanks supplied with brackish water at the hatchery facility (cohorts E-G, inclusive, Figure 5.1). At 100-110 mm length, fish were transferred to the freshwater grow-out facility, where they were maintained in 50 m^3 circular flow-through tanks (5,000-10,000 fish per tank depending upon size) supplied with geothermal artesian freshwater at 28^oC and constant aeration (cohorts A-D, inclusive, Figure 5.1). Fish were fed twice daily to satiation using a commercial pelleted feed.

A total of 62 samples from seven consecutive cohorts were taken during the winter of 2012. Ten fish from each of the cohorts A-D (inclusive) and cohort F, nine fish from cohort E and an additional three samples from a seventh cohort (cohort G) that was fertilised but yet to hatch were sampled. Total length and weight measurements were taken prior to the second gill arch on the sinistral side being sub-sampled into 10% neutral buffered formalin and a nucleic acid preservation solution (NAPS, 4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA; pH 5.5) as previously

described (111) for analyses. A unique identifier has been given to each cohort to assist with descriptions (cohort A-G, inclusive, Figure 5.1).



Cohort I.D.	G	F	E	D	C	B	A
Samples (*n*)	*n* = 3	*n* = 10	*n* = 9	*n* = 10	*n* = 10	*n* = 10	*n* = 10
Length (cm) mean (S.E.)	nd	6.8 (0.15)^{a}	12.4 (0.20)^{b}	17.6 (1.06)^{c}	20.7 (1.17)^{c}	31.9 (0.96)^{d}	32.3 (0.49)^{d}
Weight (g) mean (S.E.)	nd	4.01 (0.19)^{a}	26.1 (1.15)^{b}	88.2 (9.46)^{c}	96.0 (18.41)^{c}	415.3 (37.32)^{d}	415.0 (29.63)^{d}
Prevalence (%)	nd	90%^{c}	10%^{a,b}	30%^{a,b}	70%^{c}	50%^{b}	0%^{a}
Intensity mean (S.E.)	nd	0.42 (0.13)^{c}	0.005 (0.002)^{a}	0.006 (0.002)^{a,b}	0.02 (0.008)^{b}	0.01 (0.004)^{a,b}	0 (0)^{a}
PCR Positive (%)	1 (33%)	10 (100%)	9 (90%)	10 (100%)	10 (100%)	10 (100%)	10 (100%)
Genotype	A	A/B	B	A/B	B	B/A	B/A
Location	Hatchery	Hatchery	Hatchery	Grow-out	Grow-out	Grow-out	Grow-out

nd, no data. S.E. standard error. Superscripts (a, b, c) denote statistically significant differences between cohorts.

Figure 5.1: Timeline figure for each cohort of barramundi. Mean length (cm +/- SE), mean weight (g +/- SE), epitheliocystis prevalence (%), mean intensity (cysts/filament +/- SE), PCR positive (%), bacterial genotype and location of samples is shown. Each cohort has been designated a unique cohort I.D. (A-G inclusive).

5.3.3 Histopathology

Formalin-fixed gills were trimmed and routinely processed for histology. Paraffin-embedded gills were sectioned at 5 µm and stained with haematoxylin and eosin. Sections were examined using light microscopy to identify epitheliocystis inclusions and associated lesions (70).

5.3.4 DNA extraction, 16S rRNA amplification and sequencing

DNA was extracted from gill samples stored in NAPS using an optimised protocol for the Epicentre MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Madison, USA) (70). A broad order *Chlamydiales* PCR assay targeting the 16S rRNA gene was performed to screen each sample for the presence of the *Chlamydiales* signature sequence. The *Chlamydiales*-specific primer pair 16SIGF and

16SIGR previously designed (58) and optimised (70) was used in a 50 µL reaction including three microlitres of extracted DNA. PCR amplification reaction and cycling conditions for these assays were as previously described (70). The partial 16S rRNA sequence for the *Chlamydia*-like epitheliocystis agent was expanded with eubacterial reverse primers to 800 bp (16SIGF and 806R primer pair) and to a near full-length sequence (16SIG and 16SB1 primer pair). Cycling conditions, purification and sequencing was performed as previously described (70).

5.3.5 *In situ* hybridisation

The *Chlamydia*-like organism within the epitheliocystis cysts was detected by ISH in 5 µm serial sections. *Chlamydiales*-specific antisense (DIG*ATG TA[T/C] TAC TAA CCC TTC CGC CAC TA*DIG) and sense (DIG*ATC CTA CGC TAC TAA GTC TCT CAT CA*DIG) DIG-labeled oligonucleotide probes as described were used (24, 104) at a concentration of 10.2 pmol/mL (110). *In situ* hybridization reaction details for this assay were as previously described (110).

5.3.6 Molecular phylogenetic analysis

The 298 bp signature sequences of known *Chlamydia*-like epitheliocystis agents and the near full-length 16S rRNA regions sequenced here and data from representative *Chlamydiales* species and outgroup taxa obtained from GenBank were aligned and trimmed as previously described (70).

The software jModelTest version 0.1.1 (105) estimated TIM3+G and GTR+I+G as the best nucleotide substitution models for the signature sequence and near full-length datasets, respectively. Maximum likelihood (with 1,000 bootstraps) and Bayesian Inference (with 10,000,000 generations) analyses were performed using the software package MEGA5 (64) and Mr Bayes version 3.1.2 (95) run on the CIPRES portal (96) to explore relationships among these epitheliocystis taxa as previously described (70).

5.3.7 Quantification

Following visual inspection of the H&E stained gill sections, the prevalence (expressed as a percentage) and intensity (intensity = cysts per section/filament per section) of cysts were calculated. The presence of CLOs detected by PCR was calculated as a

prevalence (expressed as a percentage). Statistical analyses were conducted with the IBM SPSS Statistics package, version 20.0.0.1 (2011). Since the distribution of the data was non-normal (even after transformations), the non-parametric Kruskal-Wallis test with post-hoc comparisons and Mann-Whitney U test were used. A Spearman's rank correlation test was used to identify if a significant relationship existed between fish length and the prevalence of epitheliocystis.

5.3.8 Nucleotide sequence accession number

The 16S rRNA genotype sequences of the *L. calcarifer* epitheliocystis agents are available at GenBank under the accession numbers KF219613 and KF219614.

5.4 RESULTS

5.4.1 Histopathology and prevalence of novel barramundi *Chlamydia*-like organism

Mean length, mean weight, cyst prevalence (%) and intensity for each cohort are summarised in Figure 5.1. The age as days post hatch (dph) and origin of the fish sampled are also provided. There was a significant difference of fish length ($KW = 50.704$, $df = 5$, $p < 0.001$) and fish weight ($KW = 50.694$, $df = 5$, $p < 0.001$) between the cohorts (Figure 5.1). Epitheliocystis was present in all cohorts with the exception of cohort A, with prevalence as seen in histology ranging from 0 – 90% and intensity ranging from 0 – 0.42 ($\pm$ 0.13) cysts/filament. There was a significant difference in prevalence ($KW = 31.985$, $df = 5$, $p < 0.001$) and intensity ($KW = 30.519$, $df = 5$, $p < 0.001$) between the cohorts (Figure 5.1), with the greatest prevalence and intensity in fish from cohort F (Figure 5.1). Epitheliocystis was seen in all cohorts of fish except for cohort A, which were the oldest fish sampled at 514 dph. In the cohorts with epitheliocystis, hypertrophied epithelial cells were filled with membrane-enclosed cysts containing basophilic material. Clusters of cysts were seen in samples from cohort F, which were the youngest fish sampled at 87 dph (Figure 5.2 A). These cysts were found at the base of the secondary lamellae. Cysts were also seen in association with a proliferative epithelial cellular host response (Figure 5.2 B).

5.4.2 Molecular identification and phylogenetic analysis of novel *CLOs*

Initial screening by the 16S rRNA *Chlamydiales*-specific PCR assay showed that 95% (total samples tested, $n = 62$) of the barramundi samples were PCR positive for

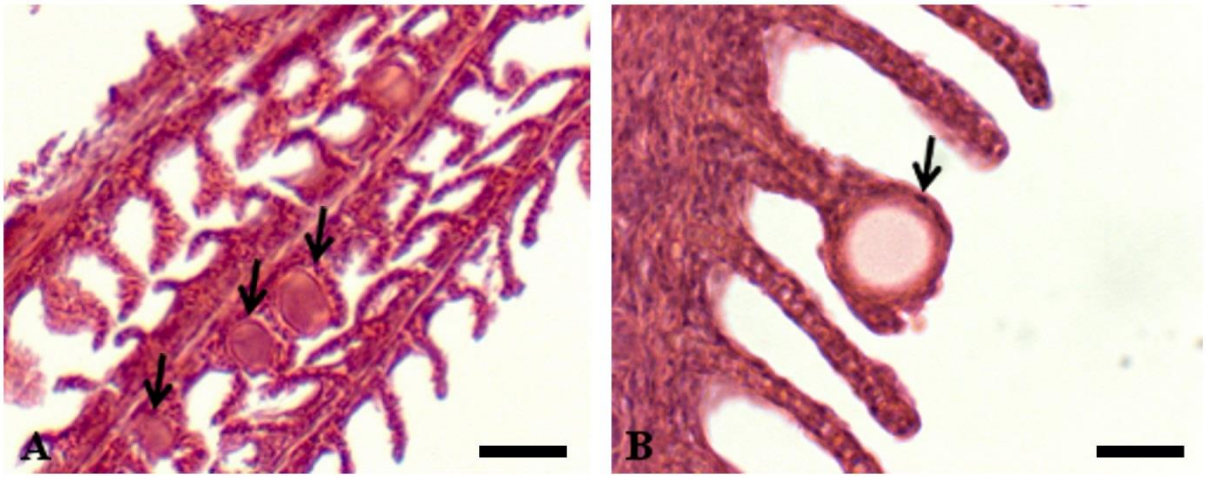


Figure 5.2: **Epitheliocystis in gills of farmed Australian Barramundi (*Lates calcarifer*) stained with haematoxylin and eosin. (A) multiple membrane-enclosed basophilic granular epitheliocystis cysts (arrows) in the gills of hatchery sized barramundi (cohort F) (scale bar = 40 µm); (B) a single membrane-enclosed cyst (arrow) with epithelial hyperplasia around the cyst and at the base of the gill filament of barramundi 320 dph (cohort C) (scale bar – 25 µm). Images are representative of host reaction across the cohorts.**

chlamydial DNA (Figure 5.1). Near full-length sequences were obtained from at least three samples per cohort (from both strands), with the exception of the pre-hatch eggs (one sample only). All PCR products were sequenced in both directions. Pairwise alignments of all near full-length sequences obtained across the cohorts revealed two distinct genotypes with 97.9% nucleotide sequence similarity to each other. A total of 29 single nucleotide polymorphisms (SNPs) were present between the two near full-length genotype sequences (1407 bp), occurring within the variable regions of the 16S rRNA gene. The SNPs were consistently found at the same positions from multiple samples within the gene when using the different primer pairs. Both genotypes were sequenced from fish of marine (cohorts E-G) and freshwater (cohorts A-D) origin (Figure 5.1).

Barramundi CLOs sequences were compared against those in the NCBI database using the BLAST-n algorithm and revealed these sequences to be novel, sharing 97.1 – 97.5% similarity to the next closest 16S rRNA sequence, *Ca*. Similichlamydia latridicola, from Australian striped trumpeter (110).

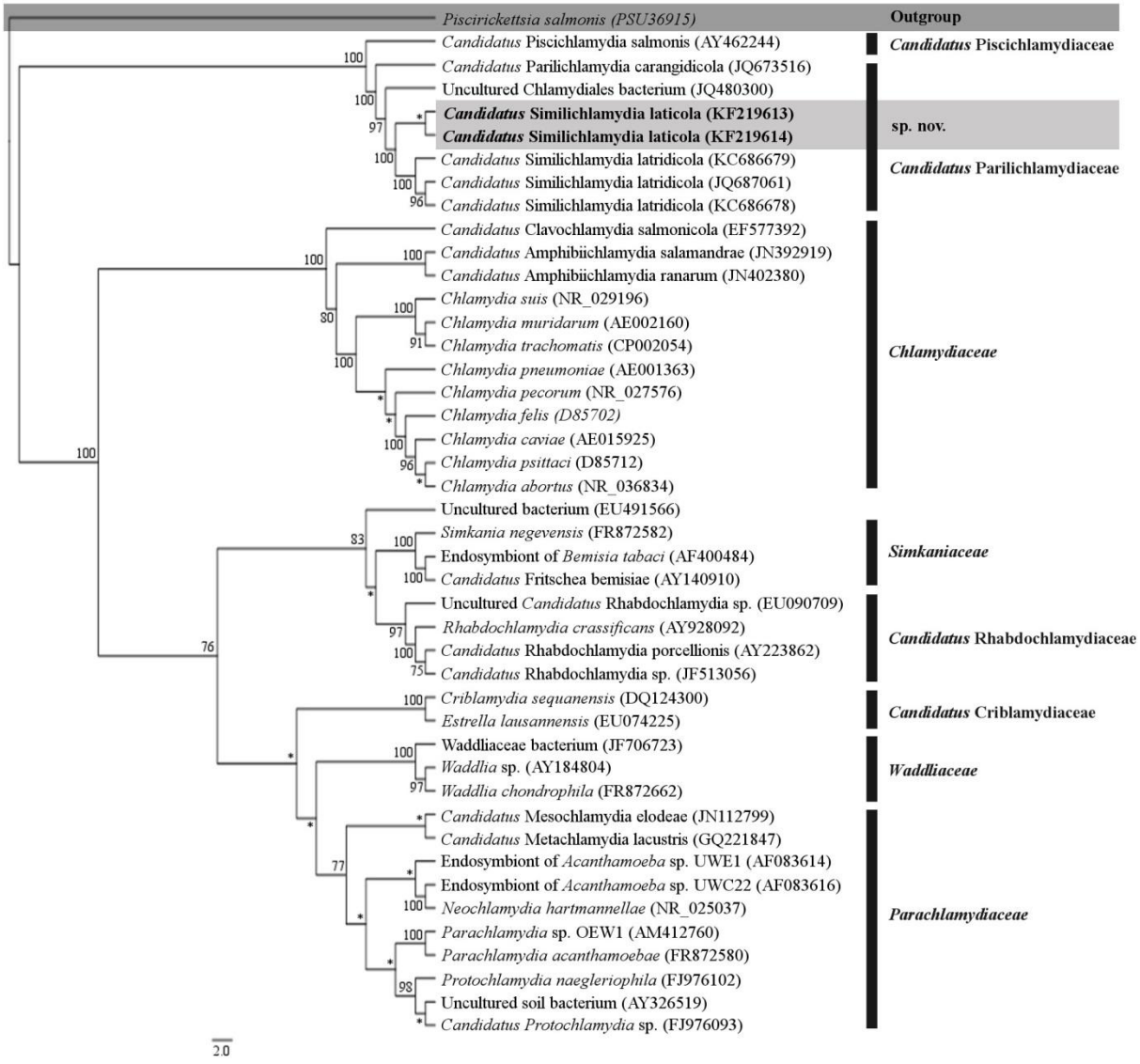


Figure 5.3: Phylogenetic relationship of '*Candidatus* Similichlamydia laticola' detected in barramundi gills with known *Chlamydiales* taxa. Branching tree based on Maximum Likelihood analysis (MEGA5) of the 16S rRNA dataset. Bootstrap support values are given at the nodes, values less that 70% are indicated by an asterisk.

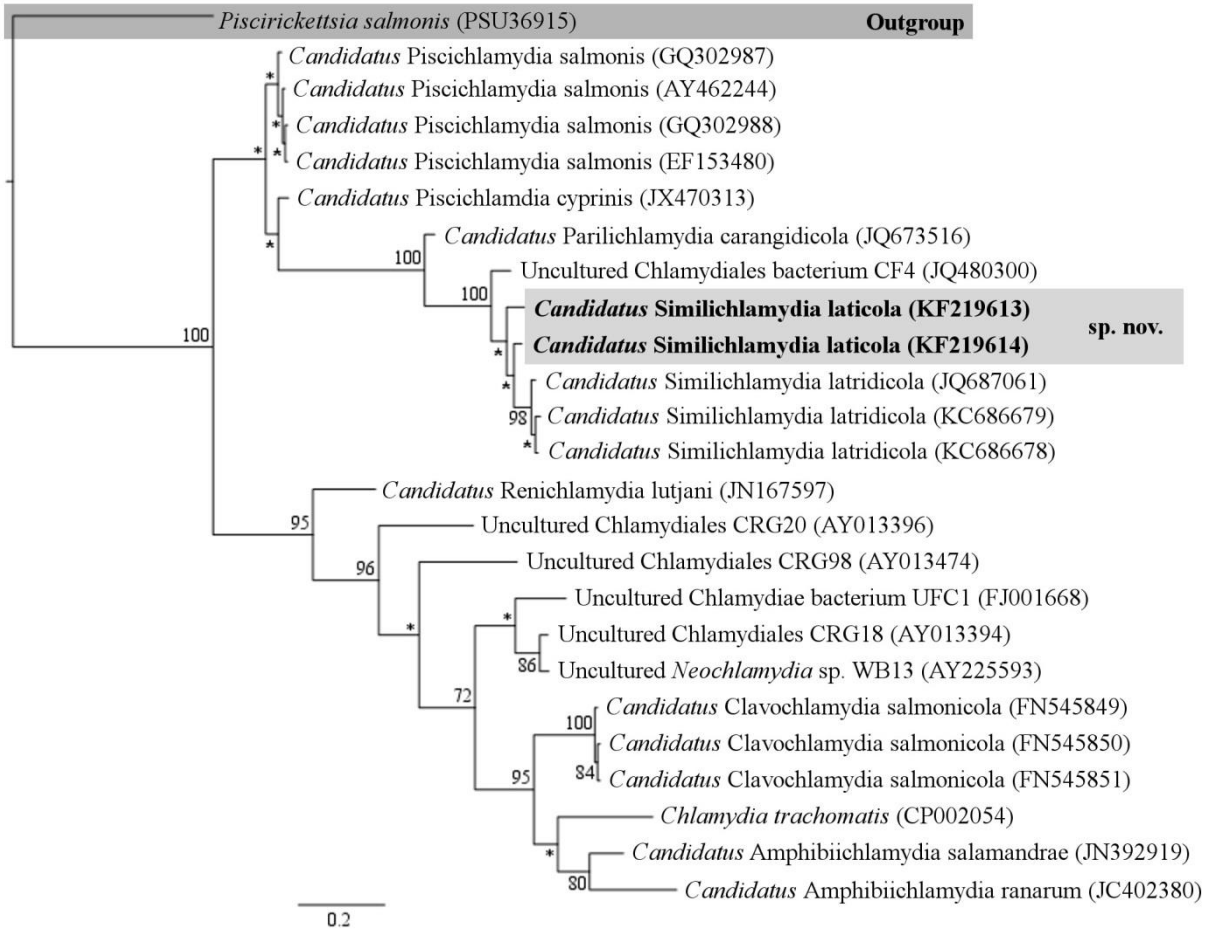


Figure 5.4: Phylogenetic relationships of the 16S rRNA signature sequences of known epitheliocystis agents. Bayesian Inference Analysis of the 16S rRNA signature sequence dataset was performed using the CIPRES portal (www.phylo.org/portal2/) (112). Posterior probabilities are given at the nodes, with values less than 70% indicated by an asterisk.

Maximum likelihood analysis of the near full-length sequence dataset yielded 1,287 characters of analysis, which resulted in a well-supported phylogram with all of the currently recognised and candidate families within the *Chlamydiales* (Figure 5.3). Bayesian inference analysis of the signature sequence dataset of known epitheliocystis agents showed that the novel CLOs reported here from barramundi grouped together in a strongly supported clade with other members of the family *Ca.* Parilichlamydiaceae and in the same genus as epitheliocystis agents of striped trumpeter, *Ca.* Similichlamydia (Figure 5.4). Phylogenetic comparisons between the sequences reported here and known epitheliocystis 16S rRNA sequences from GenBank confirm the novel species lineage of the CLOs reported here.

5.4.3 *In situ* hybridisation

The presence of CLOs in the barramundi gills was confirmed by ISH using the *Chlamydiales*-specific probes. The application of the *Chlamydiales*-specific antisense probe lead to an intense and specific labelling of the epitheliocystis inclusions within the gill epithelium (Figure 5.5 A). Sections receiving the sense probe showed no signal (Figure 5.5 B).

5.5 Discussion

In the current study, we have used several molecular methods to confirm that the agent of epitheliocystis found in barramundi gills farmed in South Australia is a novel member of the *Chlamydiales.* Phylogenetic analysis of the 16S rRNA gene showed the novel bacteria (two genotypes) from barramundi to be 97.5% similar to its nearest relative, the newly described *Ca.* Similichlamydia latridicola from striped trumpeter (110). In addition, the novel bacterium is a new species of the *Ca.* Similichlamydia genus, as per the guidelines for classification of bacteria within the order *Chlamydiales* (58).

Barramundi was the first Australian farmed species to be reported with epitheliocystis (71). Since this report, four other aquaculture species from Australia have been reported to be affected by epitheliocystis, yellowtail kingfish (17), silver perch (*Bidyanus bidyanus*) (41), Atlantic salmon (*Salmo salar*) (72) and striped trumpeter (73). While there have been no other species of fish from the family Latridae reported to

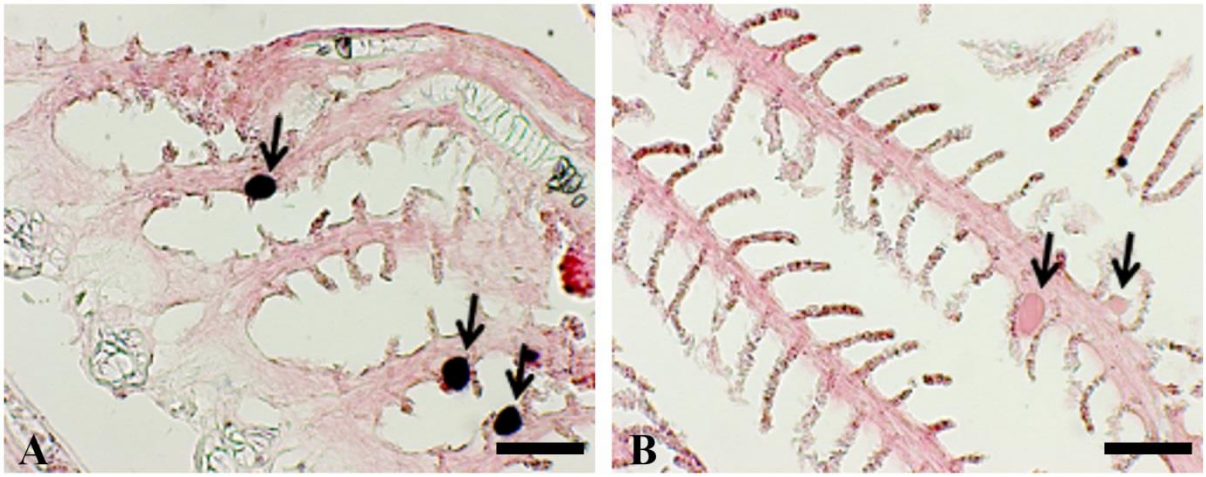


Figure 5.5: Detection of *Chlamydiales* bacteria in epitheliocystis infected gills by *in situ* hybridisation (ISH). Sections of cultured barramundi *Lates calcarifer* gill tissue tested with ISH; (A) positive reaction of epitheliocystis cysts in the gill with the antisense ISH probe, showing the purple/black colouration of the cysts (cohort F) (scale bar = 50 µm); (B) no reaction of epitheliocystis cysts in the gill with the sense ISH probe (cohort C) (scale bar = 50 µm). The images shown are representative of the staining found in other cohorts.

be affected with epitheliocystis, the condition has been reported from many species in the superfamily Percoidea, including yellowtail kingfish (70), jack mackerel (*Trachurus declivis*) (42), long-finned pike (*Dinolestes lewini*) (42), red sea bream (*Pagrus major*) (54) and silver perch (41), all of which have been reported from the Southern hemisphere.

The form of the epitheliocystis cysts observed in the barramundi of this study is in line with barramundi previously reported with this condition (71). In the previous study, 12-week old barramundi fingerlings were observed to contain hypertrophied membrane-enclosed cysts filled with a basophilic material. This previous study did not report on older fish, however it has been reported in other fish species that older, larger fish have less of a host response to epitheliocystis infections than juveniles (25). In addition, the discrepancy between negative (or low prevalence) histology and high positive PCR results has been reported previously in Atlantic salmon and yellowtail kingfish, indicating that PCR is a much more sensitive tool in detecting epitheliocystis

(27, 70). The clustering of cysts observed here is similar to the epitheliocystis hyperinfection in striped trumpeter (73). As further evidence that the epitheliocystis observed here is caused by CLOs, the order *Chlamydiales*-specific ISH probes clearly identified the *Chlamydia*-like bacteria within the epitheliocystis cysts *in situ*. The strong labelling reaction shown here is consistent with the results from both *Ca.* Piscichlamydia salmonis in farmed Atlantic salmon (57) and *Neochlamydia* sp. in farmed Arctic charr (*Salvelinus alpinus*) (35).

The detection of the CLOs from the pre-hatch eggs (cohort G) is a step forward in understanding the epidemiology and infection route of fish with epitheliocystis. There are two possible infection routes that can be determined; horizontal infection – where the infection source is coming from the environment; or vertical infection – with the infection being passed directly from the broodstock. As these fish have been maintained in captivity and were the 7th generation broodstock, held within a partially recirculating system with limited water flow and input of filtered and U.V-treated water, it is reasonable to presume that horizontal transmission is unlikely. With the detection of the CLO from the pre-hatch eggs and the multiple genotypes of CLOs sequenced from fish of both marine and freshwater origins, vertical transmission as the route of infection is plausible in this case. More data are needed to prove this hypothesis, and the development of *in vitro* culturing methods will allow researchers to confirm the infection transmission route and answer what environmental factors cause a high infection level in fish populations.

Following the guidelines for classifying bacteria in the order *Chlamydiales* (58), the current study identified two closely related but distinct bacterial sequence genotypes of the barramundi CLOs across the cohorts and sites. These CLOs did not match a previously reported CLOs from farmed Australian barramundi (24). The previously reported 298 bp signature sequence was obtained from a 14 year old archival tissue sample that was formalin-fixed and paraffin embedded. The sequence obtained was distinctly different to that reported in this study which may be due to the geographical difference of sample sources, but may also be associated with issues with the PCR amplification and sequencing of formalin-fixed and paraffin-embedded tissue samples from the previous study.

In the absence of a viable *in vitro* CLO culturing method, this study has used the molecular postulates of Fredericks and Relman (67). In summary, the combination of epitheliocystis diagnosed by standard histopathology, strong reactivity of cysts to *Chlamydiales in situ* probes and extensive phylogenetic analysis of gene sequence data from both near full-length sequences from representative *Chlamydiales* species and signature sequences from epitheliocystis agents of other fish species which have been identified across six consecutively hatched cohorts and pre-hatch eggs provides convincing evidence that the CLOs identified are the main epitheliocystis aetiological agent in farmed barramundi examined in the present study. Based on the novel 16S rRNA sequence genotypes, the percentage of sequence divergence from other *Chlamydiales* species and the observed phylogenetic relationships of the bacterial genotypes to other taxa within the order (58), the name '*Candidatus* Similichlamydia laticola' (sp. nov.) (Order *Chlamydiales*) is proposed to identify this *Chlamydia*-like agent of farmed Australian barramundi.

5.5.1 Taxonomy

"*Candidatus* Similichlamydia laticola" sp. nov., recovered from barramundi (*Lates calcarifer*). Laticola sp. nov.; la.ti'co.la. N.L. n. Lates -is, a zoological genus name; L. suff. -cola (from L. n. incola), inhabitant, dweller; N.L. n. laticola, Latis-dweller, isolated from barramundi (*Lates calcarifer*).

Obligate intracellular bacteria infecting fish gills classified as *Chlamydiales*. The new species 16S rRNA sequence is differentiated from all formally recognised and *Candidatus Chlamydiales* taxa based on a combination of morphological and genetic differences. The new species is 2.1 – 2.9% different from the 16S rRNA sequence of *Ca.* Similichlamydia placing it within this genus, but not a member of the species taxon *latridicola*, according to the classification scheme of Everett (58). Membrane-enclosed cysts present, staining basophilic under haematoxylin and eosin. Inclusions are not site specific and are found along the gill filament at the base, middle and tip of the lamellae. Inclusions react with an intense and specific labelling of the epitheliocystis inclusions when the ISH 16S rRNA *Chlamydiales* probe is applied.

5.6 ACKNOWLEDGEMENTS

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CHAPTER 6 CHLAMYDIAL INFECTIONS OF FISH: DIVERSE PATHOGENS AND EMERGING CAUSES OF DISEASE IN AQUACULTURE SPECIES

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Keywords: epitheliocystis, *Chlamydia*-like organism, fish, 16S rRNA, taxonomy, *Chlamydiales*, risk factors, transmission, diagnostics

6.1 ABSTRACT

Chlamydial infections of fish are emerging as an important cause of disease in new and established aquaculture industries. To date, epitheliocystis, a skin and gill disease associated with infection by these obligate intracellular pathogens, has been described in over 90 fish species, including hosts from marine and fresh water environments. Aided by advances in molecular detection and typing, recent years have seen an explosion in the description of these epitheliocystis-related chlamydial pathogens of fish, significantly broadening our knowledge of the genetic diversity of the order *Chlamydiales*. Remarkably, in most cases, it seems that each new piscine host studied has revealed the presence of a phylogenetically unique and novel chlamydial pathogen, providing researchers with a fascinating opportunity to understand the origin, evolution and adaptation of their traditional terrestrial chlamydial relatives. Despite the advances in this area, much still needs to be learnt about the epidemiology of chlamydial infections in fish if these pathogens are to be controlled in farmed environments. The lack of *in vitro* methods for culturing of chlamydial pathogens of fish is a major hindrance to this field. This review provides an update on our current knowledge of the taxonomy and diversity of chlamydial pathogens of fish, discusses the impact of these

infections on the health, and highlights further areas of research required to understand the biology and epidemiology of this important emerging group of fish pathogens of aquaculture species.

6.2 *CHLAMYDIA*-RELATED AGENTS OF EPITHELIOCYSTIS: AN UNDER-RECOGNISED THREAT TO AQUACULTURE INDUSTRIES?

First reported as 'Mucophilosis' in the common carp (*Cyprinus carpio*) in 1920 (23), the term 'Epitheliocystis' was coined after cyst-like inclusions within the epithelial cells of the gills were observed in the freshwater species, bluegill (*Lepomis macrochirus*) (55). Since this initial diagnosis, epitheliocystis has been reported from over 90 species of fish globally (Appendix S1) (25, 70), including fishes from marine and freshwater sources as well as from wild and farmed environments. Taxonomically, the host range for this disease is diverse, including but not limited to; [a] Acipenseridae: white sturgeon (*Acipenser trasmonanus*) (84), [b] Cyprinidae: carp (*Cyprinus carpio*) (23), [c] Salmonidae: coho salmon (*Oncorhynchus kisutch*) (33), Atlantic salmon (*Salmo salar*) (33), Arctic charr (*Salvelinus aplinus*) (35) and brown trout (*Salmo trutta*) (50), [d] Carangidae: amberjack (*Seriola dumerili*) (5), yellowtail kingfish (*S. lalandi*) (17) and yellowtail (*S. mazatlana*) (17), [e] Latidae: barramundi (*Lates calcarifer*) (24), [f] Muglidae: golden grey mullet (*Liza aurata*) (69), thinlip grey mullet (*L. ramada*) (52), greenback mullet (*L. subviridis*) (69) and striped mullet (*Mugil cephalus*) (69), [g] Terapontidae: silver perch (*Bidyanus bidyanus*) (24), and [h] Centrachidae: bluegill (*Lepomis macrochirus*) (55) and largemouth bass (*Micropterus salmoides*) (100) (Appendix S1).

Only a limited number of the affected species are cultured for commercial purposes. In these fish species, epitheliocystis is usually considered insignificant. However, there have been cases in aquaculture, such as in Atlantic salmon farmed in Norway, where epitheliocystis has been linked to high mortalities (45, 57). While its exact effect on the fish is still not fully understood, aquaculture species are more at risk of being infected due to the higher stocking densities and greater stresses placed upon the fish (16, 25). Consequently, there has been an increase in the general awareness of the impact and prevalence of this disease (25).

While the disease itself has been recognised for decades, researchers initially believed that the aetiological agent of epitheliocystis to be a single organism common to all hosts. However, as early as 1977 it was recognised that the epitheliocystis forms were a distinct taxonomic entity that demonstrated a high degree of host specificity (52). In addition, it could not be determined whether epitheliocystis was due to *Rickettsia*-like organisms (RLO) or *Chlamydia*-like organisms (CLO), due to both bacterial groups being defined as 'gram-negative, obligate and intracellular'(17, 36, 56). The identification of the actual causative agent(s) of epitheliocystis as belonging to the *Chlamydiales* was only a recent advance, primarily aided through the use of molecular techniques (57, 58).

Chlamydiales pathogens exhibit a two-stage developmental cycle of replication (58, 60). This developmental cycle rotates between (i) an extracellular infectious elementary body (EB), which is endocytosed by eukaryotic cells and resides within a cytoplasmic inclusion and; (ii) an intracellular vegetative reticulate body (RB), which replicates by binary fission (58, 60). The unique developmental cycle has hampered efforts to culture the bacteria *in vitro*. Many attempts have been made to culture the aetiological agent(s) *in vitro* as a means of proving causation and fulfilling Koch's postulates, however all have been unsuccessful to date (17, 36, 51, 55, 68).

In the absence of an *in vitro* culture system for this group of CLOs, researchers have focussed on fulfilling Fredricks and Relman's molecular postulates to establish a role for these organisms in epitheliocystis (67). Using these methods, the first CLO agent characterised in fish was *Candidatus* Piscichlamydia salmonis, which was described in Atlantic salmon in 2004 (57). Since the description of this new chlamydial species, seven new *Candidatus* species have been described with many more partially sequenced from epitheliocystis cases in different host species. Although other agents have been associated with epitheliocystis (113, 114), the majority of evidence points to a role for *Chlamydia*-related bacteria, the obligate intracellular parasites of animals and humans. Much more, however, still needs to be known about the impact of these infections on fish, their epidemiology and methods to control the disease. In this review we provide an update on the current taxonomy and diversity of *Chlamydia*-like epitheliocystis agents, discuss the impact of chlamydial infections on fish health and review the potential source and dissemination of these infections in aquaculture.

6.3 AN UPDATE OF TAXONOMY, GENETIC DIVERSITY AND GEOGRAPHICAL DISTRIBUTION OF *CHLAMYDIA*-LIKE AGENTS OF EPITHELIOCYSTIS

Bacteria belonging to the order *Chlamydiales* are an extremely important and diverse group of pathogens of vertebrates, which include respiratory diseases of fishes (60). Since the epitheliocystis review of 2006 (25), there have been numerous reports of epitheliocystis (Table 6.1). Many of these studies have focussed on molecular characterisation of the aetiological agent(s) involved and this has resulted in several new *Candidatus* species being described. It is important to note that all members of the order *Chlamydiales*, not just those associated with epitheliocystis in fish underwent a significant review in 1999, which substantially altered the taxonomy of the order (58). The taxonomic guidelines were updated to reflect the increasing use and reliance of molecular techniques and DNA sequences. Consequently, for any future bacterial isolate or sequence to be included within the order *Chlamydiales* it required a ≥ 80% sequence similarity of the 16S rRNA gene to the known and accepted members already within the order. The review went further and proposed that ≥ 90% sequence similarity conferred the same family and ≥ 95% sequence similarity conferred the same genus (58). The 95% sequence similarity criterion for genus assignment however, has been challenged by members of the *Chlamydiaceae* research community (115, 116), and researchers should use these criteria with common sense and flexibility. These criteria are also to be used for full-length 16S (or 23S) rRNA, or near full-length segments that cover all the variable domains of those genes to ensure the accuracy of the results. All new *Candidatus* sequences that have since been proposed have followed these updated taxonomic guidelines for inclusion within the order *Chlamydiales*. Currently 49 sequences associated with epitheliocystis, differentiated by PCR of the 16S rRNA regions, have been reported in the literature and/or registered with the National Centre for Biotechnology Information (NCBI) GenBank database (Figure 6.1). As the taxonomy stands today, there is no single genus of *Chlamydia*-like bacteria associated with all epitheliocystis cases (70) (Figure 6.1).

As already mentioned, the first epitheliocystis agent molecularly characterised was *Ca.* Piscichlamydia salmonis (AY462243-4) which was found in Atlantic salmon farmed in Ireland and Norway (57). These near full-length sequences were found to have ≥ 80%

Table 6.1: Cases of epitheliocystis reported since Nowak and LaPatra (2006) review. New information on aetiological agents (gene sequences) or new host species is reported.

Host Species	Geographic Origin (Environment)	Histo	ISH/FISH IHC/ICC	TEM	Gene (sequence)	Name	Length (bp)	Reference
Arctic charr (*Salvelinus alpinus*)	Canada (C)	+ve	ISH +ve	+ve	16S rRNA not submitted	nd	nd	(35)
Brown trout (*Salmo trutta*)	Norway (W)	nd	nd	nd	16S rRNA EF153480	uncultured *Chlamydiaceae* bacterium	1204	Karlsen & Nylund (GenBank)
Atlantic salmon (*Salmo salar*)	Norway (C)	+ve	ISH +ve	+ve	16S rRNA DQ011662	*Ca.* Clavichlamydia salmonicola	1294	(50)
Brown trout (*Salmo trutta*)	Norway (W)	+ve	ISH +ve	+ve	EF577392	*Ca.* Clavichlamydia salmonicola	1294	(50)
Sharpsnout sea bream (*Diplodus puntazzo*)	Greece (C)	+ve	nd	nd	nd	nd	nd	(26)
Atlantic salmon (*Salmo salar*)	Ireland (C)	+ve	nd	nd	16S cDNA FN545849-52	*Ca.* Clavichlamydia salmonicola	1244	(27)
Leopard shark (*Triakis semifasciata*)	Switzerland (Cap)	+ve	IHC -ve	nd	16S rRNA FJ001668	UFC1	294	(28)
Arctic charr (*Salvelinus alpinus*)	North America (C)	+ve	ISH +ve	+ve	16S rRNA GQ302987-88	*Ca.* Piscichlamydia salmonis	263	(49)
Brown trout (*Salmo trutta*)	Switzerland (W)	+ve	IHC +ve	+ve	16S rRNA HQ416711	*Ca.* Piscichlamydia salmonis	297	(101)
Brown trout (*Salmo trutta*)	Switzerland (W)	+ve	IHC +ve	+ve	16S rRNA HQ416712	*Ca.* Clavichlamydia salmonicola	294	(101)
Blue-striped snapper (*Lutjanus kasmira*)	Hawaii (W)	+ve*	nd	nd	16S rRNA JN167597	*Ca.* Renichlamydia lutjani	1435	(79)
Atlantic salmon (*Salmo salar*)	Norway (C)	+ve	FISH +ve	+ve	16S rRNA JN968376	*Ca.* Branchiomonas cysticola	1464	(114)
Yellowtail kingfish (*Seriola lalandi*)	Australia (C)	+ve	nd	+ve	16S rRNA JQ673516	*Ca.* Parilichlamydia carangidicola	1393	(70)
Atlantic salmon (*Salmo salar*)	Norway (C) Ireland (C)	+ve	FISH +ve	nd	16S rRNA JQ723599	*Ca.* Branchiomonas cysticola	1464	(113)
Grass carp (*Ctenopharyngodon idella*)	Austria (C)	+ve	nd	nd	16S rRNA JX470313	*Ca.* Piscichlamydia cyprinis	296	(51)
Striped trumpeter (*Latris lineata*)	Australia (C)	+ve	+ve	nd	16S rRNA JQ687061 KC686678-9	*Ca.* Similichlamydia latridicola	1396	(73, 110)
African catfish (*Clarias gariepinus*)	Uganda(C)	+ve	ISH +ve	+ve	16S rRNA JQ480299-301	*Ca.* Actinochlamydia clariae	1280	(48)
Nile tilapia (*Oreochromis nilotica*)	Uganda (nd)	nd	nd	nd	16S rRNA JQ480302-3	ON3/ON26	1410	Steigen et al. (GenBank)
Spotted eagle ray (*Aetobatus narinari*)	Florida (W)	+ve	ICC +ve	+ve	16S rRNA KC454358	UGA1	296	(117)
Rabbit fish (*Siganus canaliculatus*)	United Arab Emirates (C)	+ve	nd	nd	nd	nd	nd	(118)
Jack mackerel (*Trachurus declivis*)	Australia (W)	+ve	nd	nd	nd	nd	nd	(119)
Sand flathead (*Platycephalus bassensis*)	Australia (W)	+ve	nd	nd	nd	nd	nd	(119)
Tiger flathead (*Neoplatycephalus richardsoni*)	Australia (W)	+ve	nd	nd	nd	nd	nd	(119)
Barramundi (*Lates calcarifer*)	Australia (C)	+ve	ISH +ve	nd	16S rRNA KF219613-14	*Ca.* Similichlamydia laticola	1396	(120)

nd – no data; C – cultured; Cap – captive; W – wild

nucleotide sequence homology to members of the order *Chlamydiales*. Following the molecular taxonomic guidelines (58), this result firmly and definitively placed the epitheliocystis causative agent of Atlantic salmon within this order, and the first molecular proof that these bacteria were CLOs and not RLOs as previously speculated.

In 2008 a second aetiological agent of epitheliocystis was characterised, *Ca.* Clavochlamydia salmonicola, sequenced from both farmed Atlantic salmon (DQ011662) and wild brown trout (EF577392) from Norway (50). This bacterium was genetically distinct from the previously described *Ca*. Piscichlamydia salmonis with only 80.7% sequence similarity (from a comparison of near full-length sequences). It was also only 90.5% similar to its nearest relative, *Chlamydia abortus*, placing it within the same family but creating a new genus. As more information on this bacterium has been reported, this classification has been updated and along with a name change to *Clavichlamydia*, now stands within its own family, *Ca.* Clavichlamydiaceae. All reports of *Ca.* Clavichlamydia salmonicola have come from freshwater sourced fishes within Europe; Atlantic salmon from Ireland (FN545849-52 (27)) and Norway (EF577391 Karlsen and Nylund, unpublished and, DQ011662 (50)), and brown trout from Norway (EF577392 (50) and JN123362 Nylund, unpublished) and Switzerland (HQ416712 (101)).

Three taxonomically distinct CLOs have been reported from farmed salmonids across the Northern hemisphere (Atlantic salmon, Arctic charr and brown trout), the third being an uncultured *Neochlamydia* sp. described from Arctic charr in West Virginia, USA (35). The specific agent has never been submitted to GenBank, however it was described as being 100% identical to an uncultured clinical sample from a cat with ocular disease (AY225593 (121)).

Candidatus Parilichlamydia carangidicola (JQ673516), isolated from farmed Australian yellowtail kingfish (*Seriola lalandi*), was the first epitheliocystis agent to be characterised from the Southern hemisphere (70). This bacterium was only distantly related (87% sequence similarity) to its nearest described relative *Ca.* Piscichlamydia salmonis and formed another new family within the order, *Ca.* Parilichlamydiaceae. Not long after this case was reported, *Ca.* Actinochlamydia clariae (JQ480299-301) was

characterised from farmed African catfish (*Clarias gariepinus*) in Uganda (48). Interestingly, this bacterium was 92% similar to the agent from yellowtail kingfish placing it within the same family. These reports however, were released only three months apart and they both proposed a new family (Figure 6.1). For the purposes of this review we will use the first reported family, *Ca.* Parilichlamydiaceae. The final decision on the taxonomic nomenclature of this clade however, is yet to be decided by the '*International Committee on Systematics of Prokaryotes Subcommittee on the taxonomy of the Chlamydiae*'.

There have been three more additions to *Ca.* Parilichlamydiaceae; *Ca.* Similichlamydia latridicola from farmed and wild Australian striped trumpeter (*Latris lineata*) (JQ687061 and KC686678-9 (110)), an uncultured *Chlamydiales* bacteria from wild Nile tilapia (*Oreochromis nilotica*) in Uganda (JQ480302-3 Steigen *et al*. unpublished) and *Ca.* Similichlamydia laticola from farmed Australian barramundi (KF219613 (120)). All these epitheliocystis agents are from fish hosts exclusively sourced within the Southern hemisphere. With the evidence currently available, it is hypothesised that all the epitheliocystis agents of the *Ca.* Parilichlamydiaceae form a Southern hemisphere clade (Figure 6.1).

In 2003, a case of an 'epitheliocystis-like organism' (ELO) was reported in wild blue-striped snapper (*Lutjanus kasmira*) from Hawaii. Membrane-bound inclusions were found in internal organs and not in the gills or skin of the fish. Although the infection was found to be Gram-negative and the morphology of the ELO being somewhat comparable to epitheliocystis, the infection was seen exclusively within the kidney and spleen (122). In 2012, the agent responsible for these ELO inclusions in the wild blue-striped snapper was molecularly characterised and classified as *Ca*. Renichlamydia lutjani (JN167597 (79)). While this bacterium belongs to the order *Chlamydiales*, it is not considered to be a true epitheliocystis agent (55, 84).

Epitheliocystis does not exclusively affect teleost fishes, with two cases of CLOs being reported from cartilaginous fishes. In 2010, a novel *Chlamydiales* bacterium (FJ001668) from a leopard shark (*Triakis semifasciata*) was reported from Switzerland (28). More recently, a novel *Chlamydiales* bacterium (KC454358) from a spotted eagle ray

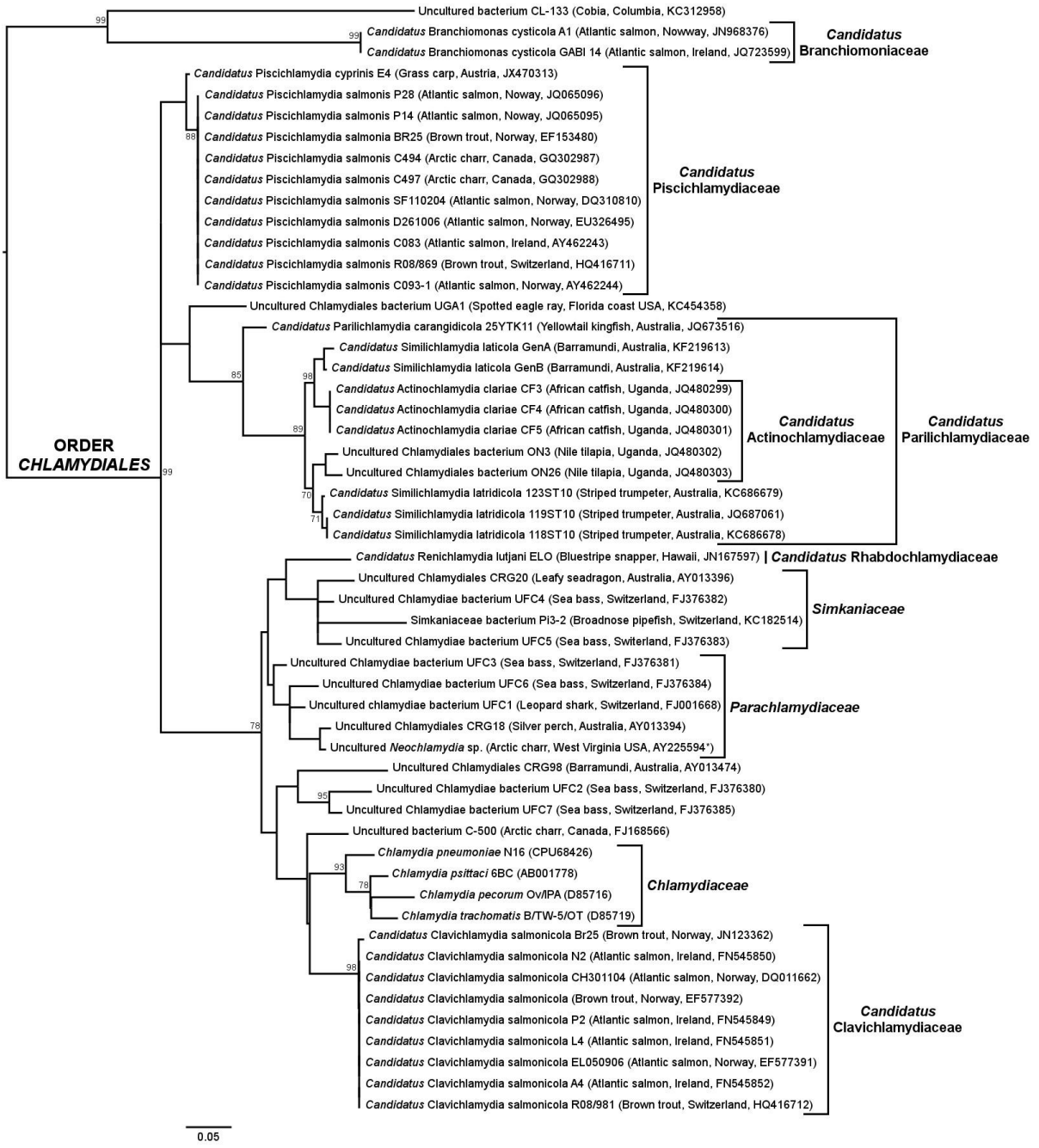

Figure 6.1: Relationships between all known *Chlamydia*-like and non-*Chlamydia*-like sequences submitted to GenBank from fishes with epitheliocystis. Origins and GenBank accession no. are given in brackets. Maximum-likelihood analysis was conducted on the 49 sequences available and four traditional *Chlamydiaceae* sequences, which were aligned and trimmed to the shortest available sequence. There were a total of 216 nucleotides in the final dataset. Nodal support was inferred by 1,000 bootstraps using MEGA5 (64), with values of less than 70% not shown.

(*Aetobatus narinari*) was reported from Florida, USA (117). The signature sequences isolated from both specimens were distantly related and shared only 80% sequence similarity (117). Both specimens were held in captivity and neither case has been named with only the short 298 bp signature sequence confirmed.

Our knowledge of the genetic diversity of CLOs associated with epitheliocystis in fish has also been expanded by the partial amplification of 16S rRNA sequences of CLOs from numerous other fish species. Molecular characterisation of archival fixed cases of epitheliocystis from Australian fish revealed the presence of three unique partial 16S rRNA sequences, CRG18 (AY013394), CRG20 (AY013396) and CRG98 (AY013474), from Australian silver perch, leafy seadragon (*Phycodurus eques*) and barramundi, respectively (24). These sequences were found to be quite distinct from each other and from those previously described (Figure 6.1), however they have not been subsequently described elsewhere.

Until recently, all epitheliocystis agents, although taxonomically diverse, fell within the order *Chlamydiales*. This changed in 2012 with the report of a novel betaproteobacterium in the gill cysts of Atlantic salmon farmed in Norway and Ireland (114). There was strong evidence that this bacterial agent could be found within the epitheliocystis cysts within the gills as shown by fluorescence *in situ* hybridisation (FISH). This was the first report of a molecularly characterised epitheliocystis bacterium that was not a CLO. The novel bacterium was named '*Ca.* Branchiomonas cysticola', and sequences from Atlantic salmon in Norway (JN968376 (114)) and Ireland (JQ723599 (113)) have now been reported (Figure 6.1).

With the ongoing descriptions of new bacterial species with each fish host investigated, questions are raised over whether this phenomenon is a reflection of the amazing diversity and host specificity of organisms within this taxonomic group, or whether these observations reflect geographic differences in the distribution of these pathogens. The geographical distribution of these agent(s) is limited. However, since its first description, *Ca.* Piscichlamydia salmonis has been detected multiple times from several species of farmed salmonids across the northern hemisphere. It has been found in epitheliocystis infected brown trout (*Salmo trutta*) from Norway (EF153480, Karlsen

and Nylund, unpublished) and Switzerland (HQ416711 (101)), Arctic charr from Canada (GQ302987-8 (49)), and further specimens of Atlantic salmon from Norway (DQ310810 (123), EU326495 (98) and JQ065095-6 (114)). There is only one report of epitheliocystis in Atlantic salmon cultured in Tasmania, Australia, and this was of very low prevalence and intensity by histology only (72). While it has been reported that *Ca*. Piscichlamydia salmonis occurs in Tasmania (45), there is no molecular evidence to support this supposition. A recent survey of Tasmanian Atlantic salmon for epitheliocystis by histology and PCR resulted in all samples being negative (Stride, Polkinghorne and Nowak, unpublished data). Although it is currently unknown what the status of epitheliocystis in farmed Atlantic salmon in Chile is, it would be worth considering. With a recent addition of *Ca.* Piscichlamydia cyprinis (JX470313 (51)), an epitheliocystis agent from grass carp (*Ctenopharyngodon idella*) farmed in Austria, there is mounting evidence that the genus *Ca*. Piscichlamydia is an agent exclusively of the northern hemisphere.

6.4 The Impact of Chlamydial Infection on Fish Health

Mortality of some farmed fish have been attributed to epitheliocystis, with rates in hatchery fish of up to 10% in largemouth bass (100) and 100% in sharpsnout sea bream (26), while adult fish have seen rates of up to 44% in Arctic charr (35) and 100% in yellowtail kingfish (Marty Deveney, Pers. Comm.). However, under what circumstances mortalities occur has yet to be determined.

There have been very few studies into the effects of epitheliocystis on fish pathology and physiology (45, 49, 51). Nevertheless, gross pathological signs are sometimes but not always observed with epitheliocystis infected fish and may include lethargy and a weak swimming behaviour, gasping at the water surface, decreased feed consumption, excessive mucous production and white nodular lesions on the gills or skin (35, 45, 53). The large surface area of the gills allows the physiological mechanisms of ammonia excretion, gas exchange, acid base balance and salt reduction to function efficiently. While fish can afford to lose approximately 50% of their respiratory function before serious issues arises (124), gill diseases, such as epitheliocystis, can cause gill pathology and impact on the efficiency and efficacy of these functions. In one of the few studies to investigate these effects further, Lai et al. (2013) recently found that serum osmolality,

which is a measure of soluble salts within the serum or plasma, was significantly increased in farmed striped trumpeter when an increase in the severity of epitheliocystis infections occurred. Infected fish had a 10% increase in their serum osmolality. In addition, chloride cells were affected by epitheliocystis infections and were absent from areas around the epitheliocystis cysts within the gill lamellae (73).

The host response to epitheliocystis presents itself in two forms, either proliferative or non-proliferative (25, 45). While epitheliocystis infections are often benign and without any proliferative host response (25, 26, 45, 70), when a host response does occur there is no consistency as to the reaction. This varied response includes, focal to multi-focal epithelial hyperplasia with interlamellar filling (28, 35, 48, 49, 51, 57, 113, 117), lamellar fusion (48, 51, 57, 113), epithelial bridging (49) and cellular hypertrophy of epithelial cells, lymphocytes or histiocytes (35, 48, 50, 101, 117). All these morphological changes occur with varying degrees of severity, ranging from mild and patchy, to diffuse and extremely severe.

Little is still known about the factors that influence the outcome of an epitheliocystis infection. One of the few studies to investigate this examined the role of the innate immune response of farmed striped trumpeter to severe epitheliocystis infections (73). They found a significant positive correlation between the severity of epitheliocystis and lysozyme activity. In fact, lysozyme activity in infected fish was 40% higher than in uninfected fish (73). Although this was the first report testing fish lysozyme levels in response to epitheliocystis infections, it has been previously tested for other bacterial infections in farmed fish, such as *Aeromonas salmonicida* in Atlantic salmon (125).

6.5 The Epidemiology of *Chlamydia*-like Epitheliocystis in Fish: More Questions than Answers?

While *Chlamydia*-like organisms can affect fish at their larval, juvenile and adult phases (25), little is still known about how each developmental phase of fish acquires their CLO and what factors influence the rate of infection and disease. Unlike RLOs, where the route of infection is known to be horizontally via fish-to-fish (126), the route of infection for CLOs is less clear. Several hypotheses exist on the potential reservoirs or vectors for CLO infection associated with epitheliocystis, including (i) vertical or horizontal

transmission; and/or (ii) the utilisation of an intermediary host such as environmental amoebae (120, 127, 128). A recent study into epitheliocystis infections in farmed barramundi found that vertical transmission was a plausible route of infection following the molecular detection of the CLO from both fertilised pre-hatch eggs and several older cohorts of fish (120). This is the first documented evidence that vertical transmission via the broodstock may play a part in transmitting the bacteria to its progeny. In terms of alternative methods of transmission, anecdotal evidence of horizontal transmission through co-infection studies using both goldfish (*Carassius auratus*) and bluegill as infection models (45) exists.

Several new species of the order *Chlamydiales* have been reported as living in association with free-living or environmental amoebae (129). It has been hypothesised that the CLOs utilise these amoebae as either symbionts or intermediary hosts (60, 127, 128). Despite this theory, and the many attempts to co-culture the CLOs with different species of amoebae (e.g. *Acanthamoeba* spp.), there have been no successful isolations of any of the fish CLOs. Most of the epitheliocystis aetiological agent(s) are host specific (24, 27, 28, 35, 48-51, 57, 60, 70, 110, 113, 114, 117, 120) and since no epitheliocystis agent has been found in association with amoebae, it seems unlikely that amoebae are the environmental reservoir of CLOs associated with epitheliocystis.

Increased stocking densities are a known risk factor for many important bacterial, viral and parasitic diseases of aquaculture species. However, the risk factors that increase the incidence of epitheliocystis infections are still speculative (25, 130). While farmed fish appear to have a greater prevalence and intensity of epitheliocystis infections over the levels experienced by wild fish (25), stocking densities alone do not appear to be the definitive risk factor for epitheliocystis infections. Even when lower stocking densities are used, for example within mesocosm-reared fish, hyperinfection of epitheliocystis still occurred (26). This suggests that another mechanism for inducing infection is occurring.

Poor water quality in the form of increased levels of pollution (118, 131) or excessive nutrients available in the water column (26), have both been attributed to increasing levels of epitheliocystis infections. More specifically, sub chronic exposure to light crude

oil induced a proliferative epithelial response with lamellae fusion and a high prevalence of epitheliocystis inclusions in juvenile rabbit fish (*Siganus canaliculatus*) after only nine days of exposure (118). Wild fish sampled around sewage outfalls, while not statistically different, also had a higher prevalence of epitheliocystis infections when compared to wild fish from 'clean' sites (74). There is also anecdotal evidence that specific nutritional deficiency within aquaculture feeds, such as a taurine deficiency in yellowtail kingfish, can induce epitheliocystis infections (Marty Deveney, Pers. Comm.).

Seasonal differences in water temperature or changes in salinity may also affect the levels of epitheliocystis infections in both farmed and wild fish. Lower water temperatures were previously linked to higher epitheliocystis infection rates in farmed amberjack (5) and striped bass (*Morone saxatilis*) (56), and wild jack mackerel (*Trachurus declivis*), sand flathead and tiger flathead (119), while higher water temperatures were associated with increased epitheliocystis infections in Australian Atlantic salmon (72). Although not yet investigated directly, salinity is thought to be a risk factor in epitheliocystis infections. Currently, there are several CLOs affecting salmonids, however only *Ca*. Clavichlamydia salmonicola has been observed in brown trout and Atlantic salmon sampled from freshwater origins. This bacterium has not been observed in any marine sourced fish and it has therefore been hypothesised that this infection ceases upon seawater transfer (27).

Narrowing down what risk factors induce and/or increase the incidence of epitheliocystis will go a long way to helping aquaculture farmers reduce morbidity and mortality of their fish. While epitheliocystis is treatable with antibiotics, this is not ideal for any fish product destined for human consumption. Epitheliocystis can be effectively treated with 25 ppm of oxytetracycline and this treatment regime was first used for infected largemouth bass (100). Interestingly, this finding was discovered by accident as the farmer believed he was treating an infection of columnaris and not epitheliocystis. Within three days, mortalities had ceased entirely with the farmer losing about 10% of his stock. Even though an effective treatment is available for epitheliocystis infections, unless a rapid, direct and non-destructive means of diagnosing epitheliocystis becomes available, diagnosis of disease outbreaks will continue to be confirmed by indirect,

destructive means. Without the means to culture the bacterium *in vitro*, future research on this area will continue to be limited.

6.6 Future Directions

Recent studies have greatly increased our knowledge of the taxonomic diversity, host range, natural source and distribution of epitheliocystis and the CLOs associated with these infections, revealing at least 30 new host species and six more characterised *Candidatus* bacterial species. While some researchers and diagnosticians believe it to be negligible when associated with fish morbidity and mortality, there is mounting evidence that it is more important to fish health than currently recognised.

Beyond the importance of these pathogens as causative agents of disease in aquaculture species, research into CLOs of epitheliocystis may be profitable for an understanding of the evolution and adaptation of pathogens of the genus *Chlamydia*, the "traditional" pathogens of humans and terrestrial animals. Inspection of the specific branching of the fish CLOs in the phylogenetic and taxonomic trees of the *Chlamydiae* indicate that fish CLOs are the oldest and deepest branched organisms within the order *Chlamydiales* (35, 48, 50, 57, 70, 79, 110, 129). As such, further research into fish CLOs may provide important insights into the origin of pathogenicity in this group of obligate intracellular pathogens, observations that have been limited thus far by study of the genomes of members of the genus *Chlamydia*, alone.

While a comparison of complete CLO genome(s) would be an important achievement in understanding the biology of fish CLOs, alongside other *Chlamydiae*, these tasks remain difficult in the absence of an *in vitro* protocol for pure culture and isolation of the causative agent(s) involved in epitheliocystis. The main problem is that CLOs do not replicate on traditional bacteriological media, and cell cultures or amoebal co-cultures are the most likely means of CLO cultivation. Despite the known protocols available for the 'traditional' *Chlamydiales* bacteria (i.e. *Chlamydia trachomatis*), these protocols have been unsuccessful in culturing CLOs from epitheliocystis infected fish. The inability to culture these CLOs *in vitro* from fish significantly hamper the development of an experimental challenge model and further studies related to the complete characterisation of these bacteria. Until this is possible, all new CLOs characterised will

remain as *Candidatus* status. The *in vitro* culture of the causative agent(s), whether they are CLOs or non-CLOs, will most likely take one of two pathways; amoebal co-culture, or host-specific cell line development. To date, two non-fish CLOs (*Waddlia chondrophila* and *Estrella lausannensis*) have been demonstrated to enter and multiply in the permanent fish cell lines EPC-175 (epithelioma papulosum cyprini cells) and RTG-2 (rainbow trout gonad cells), as well as multiple mammalian cell lines and various species of amoebae (132).

Whether the recently described CLOs of several fish species prove to be species- or region- specific requires further investigation. We know that several epitheliocystis susceptible *Seriola* species are found in multiple locations around the world including, Australia, New Zealand, Japan and Ecuador. We also know that Atlantic salmon is currently affected by three genetically distinct CLOs, all of whom occur in the northern hemisphere. It would be extremely beneficial to conduct a global survey, especially throughout Norway, Ireland, Switzerland, Canada, USA, Chile and Australia, to establish the true status (absent or present) of epitheliocystis infections, calculate its prevalence and to confirm which causative agent(s) affect farmed and wild salmonids. This work is required to map the geographical distribution of each epitheliocystis agent and see what patterns may emerge.

These intriguing pathogens should continue to be targets of aquaculture disease research and continued studies, such as those proposed, will further highlight the potential impact of these microorganisms on growing aquaculture industries while providing us with a fascinating insight into the evolution and adaptation of these obligate intracellular bacteria.

CHAPTER 7 SUMMARY AND CONCLUSION

7.1 SUMMARY OF PROJECT FINDINGS

This project was set out to investigate epitheliocystis in Australian finfish and to gain a greater understanding of the aetiological agent(s) involved. This objective was fulfilled through investigating the specific aims that were outlined in Chapter 1.

The first specific aim, *investigate and document infection levels in wild and farmed fish, and determine which factors affect epitheliocystis outbreaks*, was fulfilled in Chapter 2 for wild fish and Chapters 3 – 5, inclusive, for farmed fish. Chapter 2 reported on epitheliocystis in three commercially important wild caught species, jack mackerel, sand flathead and tiger flathead. Epitheliocystis was found in all three species over most sampling periods throughout the year. In addition, the intensity of epitheliocystis was significantly negatively correlated with water temperatures. This was the first published report of epitheliocystis in both sand flathead and tiger flathead. Chapters 3 – 5, inclusive, documented the prevalence and intensity of epitheliocystis infections in farmed yellowtail kingfish, striped trumpeter and barramundi.

The second specific aim, *characterise epitheliocystis agents from at least two species of Australian fish*, was fulfilled in Chapters 3 – 5, inclusive. Chapter 3 reported the first molecular description of an epitheliocystis aetiological agent in the Southern Hemisphere, being from yellowtail kingfish farmed in South Australia. A total of 38 samples from a period of three years were screened by conventional PCR, with randomly selected samples chosen for full-length analysis. Gene sequences across the year cohorts were identical. Through extensive phylogenetic analysis and using the guidelines by Everett et al. (1999), this novel sequence formed its own genus and species (*Ca.* Parilichlamydia carangidicola) within an undescribed *Candidatus* family (*Parilichlamydiaceae*).

Chapter 4 reported the molecular description of three novel epitheliocystis aetiological agents from farmed and wild Tasmanian striped trumpeter. Although not entirely identical, these three novel bacteria contained only six SNPs across the entire 16S rRNA gene. Phylogenetically, there was a total of only 94% sequence similarity with the

yellowtail kingfish agent described in Chapter 3. This resulted with a novel genus and species (*Ca.* Similichlamydia latridicola) within the *Candidatus* family previously described in Chapter 3.

Chapter 5 reported the molecular description of two novel epitheliocystis aetiological agents from barramundi farmed in South Australia. Samples were taken from six consecutively hatched cohorts of barramundi and also from fertilised pre-hatch eggs. The 16S rRNA gene from the novel bacteria were amplified and sequenced from all cohorts, including the pre-hatch eggs, leading to vertical transmission as a plausible route of infection in this case. The suggestion of vertical transmission had not been previously reported and is an important step towards understanding the epidemiology of epitheliocystis. Phylogenetically, the bacteria shared 97.1 – 97.5% sequence similarity to their nearest relative, previously described in Chapter 4. This resulted in a novel species (*Ca.* Similichlamydia laticola) within the *Candidatus* genus previously described in Chapter 4.

The final specific aim, *critically review the current understanding of the role of Chlamydia-like organisms found in association with epitheliocystis*, was fulfilled in Chapter 6. This review acknowledged that epitheliocystis has been recognised and diagnosed in fish for decades, occasionally reported to cause mortalities. However, the impact of these infections on the health of fish may have been underestimated. Risk factors reported in the literature that potentially increase infection prevalence and intensity include increased stocking densities, poor water quality, pollution, and seasonal changes in water temperature and salinity. While research into epitheliocystis has come a long way since this project began, there is still much more to learn.

7.2 Fulfilling Molecular Postulates

In the absence of an *in vitro* protocol, the molecular postulates of Fredricks and Relman (Table 1.4) were used to prove causation in this project. The novel aetiological agents of epitheliocystis described for yellowtail kingfish, striped trumpeter and barramundi in Chapters 3 – 5, all fulfilled five out of the seven postulates. The identical and nearly identical sequences from all three farmed species were present in all cases of disease observed by histology. The intracellular nature of these CLOs requiring a host cell to

replicate is consistent with the known biological characteristics of *Chlamydia*. The bacteria detected within the epitheliocystis cysts were confirmed by PCR of laser dissected cysts (yellowtail kingfish) or *in situ* hybridisation (striped trumpeter and barramundi). Finally, all of these results were reproducible, providing strong evidence that the epitheliocystis aetiological agents described in this thesis are of *Chlamydiales* origin.

7.3 EVOLUTION OF *CHLAMYDIALES*

At the start of this project there were only two full-length sequences available for CLOs of fish, both forming their own family (Figure 1.5). Three years on and there are now 23 unique sequences that can be split at the species level (Figure 6.1). This project alone described a new family (*Ca.* Parilichlamydiaceae), two new genera (*Ca.* Parilichlamydia and *Ca*. Similichlamydia) and three new species (*Ca*. Parilichlamydia carangidicola, *Ca*. Similichlamydia latridicola and *Ca.* Similichlamydia laticola). However, until a viable *in vitro* protocol is available and a pure culture is grown, all novel bacteria described will remain as *Candidatus* status.

Over the period of this project, not only did the diversity of the order *Chlamydiales* grow significantly, there was also a significant change for the bacteria *Ca.* Clavochlamydia salmonicola. The name of this genus was amended to *Ca.* Clavichlamydia and now it forms its own family, *Ca.* Clavichlamydiaceae, separate from the traditional family *Chlamydiaceae*. These changes were implemented by the '*International Committee on Systematics of Prokaryotes Subcommittee on the taxonomy of the Chlamydiae*' (see: http://icsp.org/subcommittee/chlamydiae/).

7.4 FUTURE DIRECTIONS

As discussed in Chapter 6, chlamydial pathogens of fish are extremely diverse and many are host specific. It would be extremely useful to gauge just how diverse these pathogens are, especially those affecting aquaculture species, by conducting global surveys to map the geographical distribution of each epitheliocystis agent. This work would help establish the true status of epitheliocystis infections, especially for farmed salmonids and *Seriola* species.

The aquaculture industry would benefit from further work into improving early diagnosis of epitheliocystis infections. Earlier detection would allow farms to manage disease outbreaks sooner, either through novel treatment or a change of husbandry practices. A better understanding of risk factors that lead to epitheliocystis outbreaks would also improve disease management on farms. For example, with evidence of vertical transmission of the disease from broodstock, a non-lethal screening method such as gill swabs or analysis of egg batches would be advantageous.

Continuing with the molecular characterisation of these CLOs would be most beneficial. Phylogenetically, these fish CLOs are the oldest and deepest branch within the order *Chlamydiales* and further research of these pathogens may lead to a greater understanding of their evolution, providing insights into the origin of their pathogenicity. The comparison of a complete genome would be an important advance in the current knowledge of *Chlamydia* and CLOs.

Finally, the largest knowledge gap in epitheliocystis research remains the inability to culture these agents *in vitro*. The intracellular nature of these pathogens requires a host cell to replicate and survive. As previously stated, many of these aetiological agents are host specific, which would most likely mean the development of specific host cell lines prior to attempting any form of *in vitro* culture. Although this may be underway in some laboratories, there are no published reports currently available.

7.5 Final Comment

This project has increased our knowledge of the host range and diversity of epitheliocystis, especially in Australian finfish. It has also led to a greater understanding of the potential route of infection by vertical transmission, with the detection of the CLO from fertilised pre-hatch barramundi eggs, which has not been previously reported. Finally, this project has contributed significantly to the taxonomy of the order *Chlamydiales* through the description of three novel species, two novel genera and one novel family.

REFERENCES

1. **FAO.** 2010. FAO Yearbook: Fishery and aquaculture statistics.
2. **Skirtun, M., P. Sahlqvist, R. Curtotti, and P. Hobsbawn.** 2012. ABARES Australian fisheries statistics 2011, Canberra, December. .
3. **Poortenaar, C., S. H. Hooker, and N. J. Sharp.** 2001. Assessment of yellowtail kingfish (*Seriola lalandi lalandi*) reproductive physiology, as a basis for aquaculture development. Aquaculture **201:**271-286.
4. **Benetti, D. D.** 1997. Spawning and larval husbandry of flounder (*Paralichthys woolmani*) and Pacific yellowtail (*Seriola mazatlana*), new candidate species for aquaculture. Aquaculture **155:**307-318.
5. **Crespo, S., A. Grau, and F. Padros.** 1990. Epitheliocystis disease in the cultured amberjack, *Seriola dumerili* Risso (Carangidae). Aquaculture **90:**197-207.
6. **Egusa, S.** 1983. Disease problems in Japanese yellowtail, *Seriola quinqueradiata* culture: a review. Rapports et Proces-verbaux des Réunions. Conseil International pour l'Éxploration de la Mer **182:**10-18.
7. **Grau, A., S. Crespo, E. Pastor, P. Gonzalez, and E. Carbonell.** 2003. High infection by *Zeuxapta seriolae* (Monogenea: Heteraxinidae) associated with mass mortalities of amberjack *Seriola dumerili* Risso reared in sea cages in the Balearic Islands (western Mediterranean). Bulletin of the European Association of Fish Pathologists **23:**139-142.
8. **Montero, F. E., S. Crespo, F. Padros, F. De la Gandara, A. Garcia, and J. A. Raga.** 2004. Effects of the gill parasite *Zeuxapta seriolae* (Monogenea: Heterxinidae) on the amberjack *Seriola dumerili* Risso (Teleostei: Carangidae). Aquaculture **232:**153-163.
9. **Sharp, N. J., B. K. Diggles, C. W. Poortenaar, and T. J. Willis.** 2004. Efficacy of Aqui-S, formalin and praziquantel against the monogeneans, *Benedenia seriolae* and *Zeuxapta seriolae*, infecting yellowtail kingfish *Seriola lalandi lalandi* in New Zealand. Aquaculture **236:**67-83.
10. **Mansell, B., M. D. Powell, I. Ernst, and B. F. Nowak.** 2005. Effects of the gill monogenean *Zeuxapta seriolae* (Meserve, 1938) and treatment with hydrogen peroxide on pathophysiology of kingfish, *Seriola lalandi* Valenciennes, 1833. Journal of Fish Diseases **28:**253-262.

11. **Hutson, K. S., I. Ernst, and I. D. Whittington.** 2007. Risk assessment for metazoan parasites of yellowtail kingfish *Seriola lalandi* (Perciformes : Carangidae) in South Australian sea-cage aquaculture. Aquaculture **271:**85-99.
12. **Williams, R. E., I. Ernst, C. B. Chambers, and I. D. Whittington.** 2007. Efficacy of orally administered praziquantel against *Zeuxapta seriolae* and *Benedenia seriolae* (Monogenea) in yellowtail kingfish *Seriola lalandi*. Diseases of Aquatic Organisms **77:**199-205.
13. **Leef, M. J., and P. S. Lee.** 2009. Preliminary investigation into the killing effect of kingfish (*Seriola lalandi*) serum and mucus against the monogenean parasites *Benedenia seriolae* and *Zeuxapta seriolae*. Aquaculture International **17:**607-614.
14. **Stephens, F. J., and A. Savage.** 2010. Two mortality events in sea-caged yellowtail kingfish *Seriola lalandi* Valenciennes, 1833 (Nannopercidae) from Western Australia. Australian Veterinary Journal **88:**414-416.
15. **Kawakami, H., H. Yamashita, and M. Sakai.** 2000. Comparative sensitivity of yellowtail *Seriola quinqueradiata* and goldstriped amberjack *S. aureovittata* (*S. lalandi*) to *Photobacterium damsela* subsp. *piscicida*. Journal of the World Aquaculture Society **31:**213-217.
16. **Grau, A., and S. Crespo.** 1991. Epitheliocystis in the wild and cultured amberjack, *Seriola dumerili* Risso: Ultrastructural observations. Aquaculture **95:**1-6.
17. **Venizelos, A., and D. D. Benetti.** 1996. Epitheliocystis disease in cultured yellowtail *Seriola mazatlana* in Ecuador. Journal of the World Aquaculture Society **27:**223-227.
18. **Froese, R., and D. Pauly** 2011, posting date. FishBase. Family Latridae - Trumpeters. [Online.]
19. **Battaglene, S. C., and J. M. Cobcroft.** 2007. Advances in the culture of striped trumpeter larvae: A review. Aquaculture **268:**195-208.
20. **Hartmann, K., and J. M. Lyle.** 2011. Tasmanian scalefish fishery - 2009/10. Institute for Marine and Antarctic Studies, University of Tasmania.
21. **Battaglene, S. C., and J. M. Cobcroft.** 2010. Enhanced hatchery production of striped trumpeter, *Latris lineata*, in Tasmania through system design, microbial control and early weaning. Aquafin CRC.
22. **Schipp, G., J. Bosmans, and J. Humphrey.** 2007. Northern Territory barramundi farming handbook. Department of Resources, Northern Territory Government.

23. **Plehn, M.** 1920. Praktikum der Fischkrankheiten. In: Demoll R, Maier H (eds). Handbuch der Binnenfischerei Mitteleuropas. E. Schweyerbart, Stuttgart:p 301-429.

24. **Meijer, A., P. J. M. Roholl, J. M. Ossewaarde, B. Jones, and B. F. Nowak.** 2006. Molecular evidence for association of *Chlamydiales* bacteria with epitheliocystis in Leafy seadragon (*Phycodurus eques*), Silver perch (*Bidyanus bidyanus*), and Barramundi (*Lates calcarifer*). Applied and Environmental Microbiology **72:**284-290.

25. **Nowak, B. F., and S. E. LaPatra.** 2006. Review: Epitheliocystis in fish. Journal of Fish Diseases **29:**573-588.

26. **Katharios, P., M. Papadaki, N. Papandroulakis, and P. Divanach.** 2008. Severe mortality in mesocosm-reared sharpsnout sea bream *Diplodus puntazzo* larvae due to epitheliocystis infection. Diseases of Aquatic Organisms **82:**55-60.

27. **Mitchell, S. O., T. Steinum, H. Rodger, C. Holland, K. Falk, and D. J. Colquhoun.** 2010. Epitheliocystis in Atlantic salmon, *Salmo salar* L., farmed in fresh water in Ireland is associated with '*Candidatus* Clavochlamydia salmonicola' infection. Journal of Fish Diseases **33:**665-673.

28. **Polkinghorne, A., H. Schmidt-Posthaus, A. Meijer, A. Lehner, and L. Vaughan.** 2010. Novel *Chlamydiales* associated with epitheliocystis in a leopard shark *Triakis semifasciata*. Diseases of Aquatic Organisms **91:**75-81.

29. **Kent, M. L., G. S. Traxler, D. Kieser, J. Richard, S. C. Dawe, R. W. Shaw, G. Prosperi-Porta, J. Ketcheson, and T. P. T. Evelyn.** 1998. Survey of Salmonid pathogens in ocean-caught fishes in British Columbia, Canada. Journal of Aquatic Animal Health **10:**211-219.

30. **Whittington, R. J., J. B. Jones, P. M. Hine, and A. D. Hyatt.** 1997. Epizootic mortality in the pilchard *Sardinops sagax neopilchardus* in Australia and New Zealand in 1995. I. Pathology and epizoology. Diseases of Aquatic Organisms **28:**1-16.

31. **Desser, S., W. Paterson, and D. Steinhagen.** 1988. Ultrastructure observations on the causative agent of epitheliocysits in the brown bullhead, *Ictalurus nebulosus* Lesueur, from Ontario and a comparison with the chlamydiae of higher vertebrates. Journal of Fish Diseases **11:**453-460.

32. **Zimmer, M. A., M. S. Ewing, and K. M. Kocan.** 1984. Epitheliocystis disease in the channel catfish, *Ictalurus punctatus* (Rafinesque). Journal of Fish Diseases **7:**407-410.

33. **Carvajal, J., G. Ruiz, and L. Gonzalez.** 1990. Gill histopathologies in coho salmon (*Oncorhynchus kisutch*) and Atlantic salmon (*Salmo salar*) raised under net cages in southern Chile. Medio ambiente. Valdivia **11:**53-58.

34. **Rourke, A. W., R. W. Davis, and T. M. Bradley.** 1984. A light and electron microscope study of epitheliocystis in juvenile steelhead trout, *Salmo gairdneri* Richardson. Journal of Fish Diseases **7:**301-309.

35. **Draghi, A., II, J. Bebak, V. L. Popov, A. C. Noble, S. J. Geary, A. West, P. Byrne, and S. Frasca, Jr.** 2007. Characterization of a *Neochlamydia*-like bacterium associated with epitheliocystis in cultured Arctic charr *Salvelinus alpinus*. Diseases of Aquatic Organisms **76:**27-38.

36. **Bradley, T. M., C. E. Newcomer, and K. O. Maxwell.** 1988. Epitheliocystis associated with massive mortalities of cultured lake trout *Salvelinus namaycush*. Diseases of Aquatic Organisms **4:**9-17.

37. **Langdon, J. S., K. Elliott, and B. MacKay.** 1991. Epitheliocystis in the leafy sea-dragon. Australian Veterinary Journal **68:**244-244.

38. **Longshaw, M., M. J. Green, and S. W. Feist.** 2004. Histopathology of parasitic infections in greater pipefish, *Syngnathus acus* L., from an estuary in the UK. Journal of Fish Diseases **27:**245-248.

39. **Paperna, I., and F. Baudin-Laurencin.** 1979. Parasitic infections of sea bass, *Dicentrarchus labrax*, and gilt head sea bream, *Sparus aurata*, in mariculture facilities in France. Aquaculture **16:**173-175.

40. **Wolke, R. E., D. S. Wyand, and L. H. Khairallah.** 1970. A light and electron microscopic study of epitheliocystis disease in the gills of Connecticut striped bass (*Morone saxatilis*) and white perch (*Morone americanus*). Journal of Comparative Pathology **80:**559-563.

41. **Frances, J., R. Tennent, and B. F. Nowak.** 1997. Epitheliocystis in silver perch, *Bidyanus bidyanus* (Mitchell). Journal of Fish Diseases **20:**453-457.

42. **Nowak, B. F., D. Dawson, L. Basson, M. Deveney, and M. D. Powell.** 2004. Gill histopathology of wild marine fish in Tasmania: potential interactions with gill

health of cultured Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases **27:**709-717.

43. **Tricklebank, K. A.** 1997. Organochlorine pesticides residues and histopathology in the Damselfish *Parma microlepsis*, from reefs in New South Wales. PhD Thesis. University of Sydney, Sydney.
44. **Miyazaki, T., Y. Fujimaki, and K. Hatai.** 1986. A light and electron microscopic study on epitheliocystis disease in cultured fishes. Nippon Suisan Gakkaishi **52:**199-202.
45. **Mitchell, S. O., and H. D. Rodger.** 2011. A review of infectious gill disease in marine salmonid fish. Journal of Fish Diseases **34:**411-432.
46. **Lewis, E. J., S. M. McLaughlin, J. E. Bodammer, and T. K. Sawyer.** 1992. Epitheliocystis in ten new host species of marine fish. Journal of Fish Diseases **15:**267-271.
47. **Crespo, S., C. Zarza, F. Padros, and M. Marin de Mateo.** 1999. Epitheliocystis agents in sea bream *Sparus aurata*: morphological evidence for two distinct *Chlamydia*-like developmental cycles. Diseases of Aquatic Organisms **37:**61-72.
48. **Steigen, A., A. Nylund, E. Karlsbakk, P. Akoll, I. U. Fiksdal, S. Nylund, R. Odong, H. Plarre, R. Semyalo, C. Skar, and K. Watanabe.** 2013. '*Cand*. Actinochlamydia clariae' *gen. nov., sp. nov.,* a unique intracellular bacterium causing epitheliocystis in Catfish (*Clarias gariepinus*) in Uganda. PLoS One **8:**e66840.
49. **Draghi, A., J. Bebak, S. Daniels, E. R. Tulman, S. J. Geary, A. West, V. L. Popov, and S. Frasca Jr.** 2010. Identification of '*Candidatus* Piscichlamydia salmonis' in Arctic charr *Salvelinus alpinus* during a survey of charr production facilities in North America. Diseases of Aquatic Organisms **89:**39-49.
50. **Karlsen, M., A. Nylund, K. Watanabe, J. V. Helvik, S. Nylund, and H. Plarre.** 2008. Characterisation of '*Candidatus* Clavochlamydia salmonicola': an intracellular bacterium infecting salmonid fish. Environmental Microbiology **10:**208-218.
51. **Kumar, G., R. Mayrhofer, G. Soliman, and M. El-Matbouli.** 2012. Novel *Chlamydiales* associated with epitheliocystis in grass carp (*Ctenopharyngodon idella*). Veterinary Record **172**.
52. **Paperna, I.** 1977. Epitheliocystis infection in wild and cultured sea bream (*Sparus aurata*, Sparidae) and grey mullets (*Liza ramada*, Mugilidae). Aquaculture **10:**169-176.

53. **Nylund, A., A. M. Kvenseth, and E. Isdal.** 1998. A morphological study of the epitheliocystis agent in farmed Atlantic salmon. Journal of Aquatic Animal Health **10:**43-55.

54. **Syasina, I., I. S. Park, and J. M. Kim.** 2004. Epitheliocystis disease in red sea bream *Pagrus major* and induced hybrid, red sea bream *Pagrus major* female black sea bream *Acanthopagrus schlegeli* male, cultured in Korea. Bulletin of the European Association of Fish Pathologists **24:**260-267.

55. **Hoffman, G., C. E. Dunbar, K. Wolf, and L. O. Zwillenberg.** 1969. Epitheliocystis, a new infectious disease of Bluegill (*Lepomis macrouchirus*). Antonie van leeuwenhoek **35:**146-158.

56. **Zachary, A., and I. Paperna.** 1977. Epitheliocystis disease in the striped bass *Morone saxatilis* from the Chesapeake Bay. Canadian Journal of Microbiology **23:**1404-1414.

57. **Draghi, A., V. L. Popov, M. M. Kahl, J. B. Stanton, C. C. Brown, G. J. Tsongalis, A. West, and S. Frasca.** 2004. Characterization of '*Candidatus Piscichlamydia salmonis*' (Order *Chlamydiales*), a *Chlamydia*-like bacterium associated with epitheliocystis in farmed Atlantic Salmon (*Salmo salar*). Journal of Clinical Microbiology **42:**5286-5297.

58. **Everett, K. D. E., R. M. Bush, and A. A. Anderson.** 1999. Emended description of the order *Chlamydiales*, proposal of *Parachlamydiceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiceae*, including a new genus and five new species, and standards for the identification of organisms. International Journal of Systematic Bacteriology **49:**415-440.

59. **Ossewaarde, J. M., and A. Meijer.** 1999. Molecular evidence for the ecistence of additional members of the order *Chlamydiales*. Microbiology **145:**411-417.

60. **Corsaro, D., and G. Greub.** 2006. Pathogenic potential of novel *Chlamydiae* and diagnostic approaches to infections due to these obligate intracellular bacteria. Clinical Microbiology Reviews **19:**283-297.

61. **Corsaro, D., M. Valassina, and D. Venditti.** 2003. Increasing diversity within *Chlamydiae*. Critical Reviews in Microbiology **29:**37-78.

62. **Fryer, J. L., and C. N. Lannan.** 1994. Rickettsial and chlamydial infections of fresh-water and marine fishes, bivalves, and crustaceans. Zoological Studies **33:**95-107.

63. **Greub, G., and D. Raoult.** 2002. Crescent bodies of *Parachlamydia acanthamoeba* and its life cycle within *Acanthamoeba polyphaga*: an electron micrograph study. Applied and Environmental Microbiology **68:**3076-3084.

64. **Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar.** 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. Molecular Biology and Evolution **28:**2731-2739.

65. **Evans, A. S.** 1976. Causation and disease: The Henle-Koch postulates revisited. The Yale Journal of Biology and Medicine **49:**175-195.

66. **Evans, A. S.** 1991. Causation and disease: effect of technology on postulates of causation. The Yale Journal of Biology and Medicine **64:**513-528.

67. **Fredricks, D. N., and D. A. Relman.** 1996. Sequence-based identification of microbial pathogens: a recondiseration of Koch's postulates. Clinical Microbiology Reviews **9:**18-33.

68. **Valdron, K. A., D. K. Cone, and R. Cusack.** 1994. Persistent nodular lesions of bacterial origin on the gills of gasterosteid fish inhabiting an estuarine bay in Nova Scotia, Canada. Journal of Fish Diseases **17:**673-679.

69. **Paperna, I., and I. Sabnai.** 1980. Epitheliocystis disease in fishes. In: Fish diseases. Third COPRAQ-Session., Springer-Verlag Berlin, Heidelberg.

70. **Stride, M. C., A. Polkinghorne, T. L. Miller, J. M. Groff, S. E. LaPatra, and B. F. Nowak.** 2013. Molecular characterisation of '*Candidatus* Parilichlamydia carangidicola', a novel *Chlamydia*-like epitheliocystis agent in Yellowtail kingfish, *Seriola lalandi* (Valenciennes), and the proposal of a new family, '*Candidatus* Parilichlamydiaceae' fam. nov. (Order *Chlamydiales*). Applied and Environmental Microbiology **79:**1590-1597.

71. **Anderson, I., and H. C. Prior.** 1992. Subclinical epitheliocystis in barramundi, *Lates calcarifer* , reared in sea cages. Australian Veterinary Journal **69:**226-227.

72. **Nowak, B. F., and A. Clark.** 1999. Prevalence of epitheliocystis in Atlantic salmon, *Salmo salar* L., farmed in Tasmania, Australia. Journal of Fish Diseases **22:**73-78.

73. **Lai, C. C., P. B. B. Crosbie, S. C. Battaglene, and B. F. Nowak.** 2013. Effects of epitheliocystis on serum lysozyme activity and osmoregulation in cultured juvenile striped trumpeter *Latris lineata* (Forster). Aquaculture **388-391:**99-104.

74. **Nowak, B. F.** 1996. Health of Red Morwong, *Cheilodactylus fuscus*, and Rock Cale, *Crinodus lophodon*, from Sydney Cliff-face sewage outfalls. Marine Pollution Bulletin **33:**281-292.

75. **Miyaki, K., K. Mizuta, N. Yamamoto, K. Yoshikoshi, K. Kanai, and O. Tabeta.** 1998. Mass mortality of hatchery-reared juveniles of bartail flathead, *Platycephalus* sp. due to epitheliocystis-like disease. Bulletin of Nagasaki Prefectural Institute of Fisheries **24:**7-10.

76. **MacLean, S. A., and L. A. Fuiman.** 1993. Pathological conditions of Narragansett Bay young-of-the-year winter flounder. Water quality and the early life stages of fishes, American Fisheries Society Symposium **14:**47-54.

77. **Munday, B. L., and D. G. Brand.** 1992. Apparently - embolic, enigmatic bodies in the gill filaments of fish. Bulletin of the European Association of Fish Pathologists **12:**127-130.

78. **Heidel, J. R., B. F. Nowak, K. A. Fischer, V. G. Watral, and M. L. Kent.** 2002. Visceral nodular cartilaginous metaplasia in rockfishes (*Sebastes* spp.) in the eastern North Pacific Ocean. Journal of Veterinary Diagnostic Investigation **14:**495-497.

79. **Corsaro, D., and T. M. Work.** 2012. *Candidatus* Renichlamydia lutjani, a Gram-negative bacterium in internal organs of blue-striped snapper *Lutjanus kasmira* from Hawaii. Diseases of Aquatic Organisms **98:**249-254.

80. **Delgado, C. L., N. Wada, M. W. Rosegrant, S. Meijer, and M. Ahmed.** 2003. Outlook for fish to 2020: meeting global demand. International Food Policy Research Institute.

81. **Jover, M., A. Garcia-Gomez, A. Tomas, F. De la Gandara, and L. Perez.** 1999. Growth of mediterranean yellowtail (*Seriola dumerilii*) fed extruded diets containing different levels of protein and lipid. Aquaculture **179:**25-33.

82. **Cobcroft, J. M., P. M. Pankhurst, C. Poortenaar, B. Hickman, and M. Tait.** 2004. Jaw malformation in cultured yellowtail kingfish (*Seriola lalandi*) larvae. New Zealand Journal of Marine and Freshwater Research **38:**67-71.

83. **Sharp, N. J., C. W. Poortenaar, B. K. Diggles, and T. J. Willis.** 2003. Metazoan parasites of yellowtail kingfish, *Seriola lalandi lalandi*, in New Zealand: prevalence, intensity, and site preference. New Zealand Journal of Marine and Freshwater Research **37:**273-282.

84. **Groff, J. M., S. E. LaPatra, R. J. Munn, M. L. Anderson, and B. I. Osburn.** 1996. Epitheliocystis infection in cultured white sturgeon, *Acipenser transmontanus*: antigenic and ultrastructural similarities of the causative agent to the *Chlamydiae*. Journal of Veterinary Diagnostic Investigation **8:**172-180.

85. **Kim, D. J., J. H. Park, S. H. Seok, S. A. Cho, M. W. Baek, and H. Y. Lee.** 2005. Epitheliocystis in Carp (*Cyprinus carpio*) in South Korea. Journal of Veterinary Medical Science **67:**119-120.

86. **Draghi, A., II.** 2006. Molecular characterization of *Chlamydia*-like bacteria associated with epitheliocystis in farmed salmonids, Atlantic salmon (*Salmo salar*) and Arctic char (*Salvelinus alpinus*), and environmental *Chlamydiae* from aquatic environments. PhD Thesis. University of Connecticut.

87. **Relman, D. A.** 1993. Universal bacterial 16S rDNA amplification and sequencing, p. 489-495. *In* D. H. Pershing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), Diagnostic molecular microbiology: principles and applications., Washington DC.

88. **Hosokawa, T., Y. Kikuchi, N. Nikoh, M. Shimada, and T. Fukatsu.** 2006. Strict host-symbiont cospeciation and reductive genome evolution in insect gut bacteria. PLoS Biology **4:**1841-1851.

89. **Drummond, A. J., B. Ashton, S. Buxton, M. Cheung, A. Cooper, C. Duran, M. Field, J. Heled, M. Kearse, S. Markowitz, R. Moir, S. Stones-Havas, S. Sturrock, T. Thierer, and A. Wilson.** 2011. Geneious v5.4, Available from http://www.geneious.com/.

90. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSIBLAST: a new generation of protein database search programs. Nucleic Acids Research **25:**3389-3402.

91. **Edgar, R. C.** 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research **32:**1792-1797.

92. **Maddison, W. P., and D. R. Maddison.** 2009. Mesquite: a modular system for evolutionary analysis. Version 2.72 http://mesquiteproject.org/.

93. **Guindon, S., and O. Gascuel.** 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology **52:**696-704.

94. **Posada, D.** 2008. jModelTest: Phylogenetic Model Averaging. Molecular Biology and Evolution **25:**1253-1256.

95. **Ronquist, F., and J. P. Huelsenbeck.** 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19:**1572-1574.

96. **Miller, M. A., M. T. Holder, R. Vos, P. E. Midford, T. Liebowitz, L. Chan, P. Hoover, and T. Warnow.** 2009. The CIPRES Portals. CIPRES. 2009-08-04. URL:http://www.phylo.org/sub_sections/portal. Accessed: 2009-08-04. (Archived by WebCite(r) at http://www.webcitation.org/5imQlJeQa).

97. **Stamatakis, A., P. Hoover, and J. Rougemont.** 2008. A rapid bootstrap algorithm for the RAxML web servers. Systematic Biology **57:**758-771.

98. **Nylund, A., K. Watanabe, S. Nylund, M. Karlsen, P. A. Saether, C. E. Arnesen, and E. Karlsbakk.** 2008. Morphogenesis of salmonid gill poxvirus associated with proliferative gill disease in farmed Atlantic salmon (*Salmo salar*) in Norway. Archives in Virology **153:**1299-1309.

99. **Costa, G., J. Lom, C. Andrade, and R. Barradas.** 1998. First report of *Ceratomyxa sparusaurati* (Protozoa: Myxosporea) and the occurrence of epitheliocystis in cultured sea bream, *Sparus aurata* L. from Madeira. Bulletin of the European Association of Fish Pathologists **18:**165-167.

100. **Goodwin, A. E., E. Park, and B. F. Nowak.** 2005. Short communication: Successful treatment of largemouth bass, *Micropterus salmoides* (L.), with epitheliocystis hyperinfection. Journal of Fish Diseases **28:**623-625.

101. **Schmidt-Posthaus, H., A. Polkinghorne, L. Nufer, A. Schifferli, D. R. Zimmermann, H. Segner, P. Steiner, and L. Vaughan.** 2012. A natural freshwater origin for two chlamydial species, *Candidatus* Piscichlamydia salmonis and *Candidatus* Clavochlamydia salmonicola, causing mixed infections in wild brown trout (*Salmo trutta*). Environmental Microbiology **14:**2048-2057.

102. **Cobcroft, J. M., and S. C. Battaglene.** 2013. Ultraviolet irradiation is an effective alternative to ozonation as a seawater treatment to prevent Kudoa neurophila infection of striped trumpeter. Journal of Fish Diseases **369:**57-65.

103. **Andrews, M., S. C. Battaglene, J. M. Cobcroft, M. B. Adams, E. J. Noga, and B. F. Nowak.** 2010. Host response to the chondracanthid copepod *Chondracanthus goldsmidi*, a gill parasite of the striped trumpeter, *Latris lineata* (Forster), in Tasmania. Journal of Fish Diseases **33:**211-220.

104. **Meijer, A., J. A. van der Vliet, P. J. M. Roholl, S. K. Gielis-Proper, A. de Vries, and J. M. Ossewaarde.** 1999. *Chlamydia pneumoniae* in abdominal aortic aneurysms:

abundance of membrane components in the absence of Heat Shock Protein 60 and DNA. Arteriosclerosis, Thrombosis, and Vascular Biology **19:**2680-2686.

105. **Posada, D.** 2008. jModelTest: Phylogenetic Model Averaging. Mol. Biol. Evol. **25:**1253-1256.
106. **Guindon, S., and O. Gascuel.** 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. **52:**696-704.
107. **Szakolczai, J., F. Vetesi, and Pitz.** 1999. Epitheliocystis Disease in Cultured Pacu (*Piaractus mesopotamicus*) in Brazil. Acta Veterinaria Hungarica **47:**311-318.
108. **Company, R., A. Sitja-Bobadilla, M. J. Pujalte, E. Garay, P. Alvarez-Pellitero, and J. Perez-Sanchez.** 1999. Bacterial and parasitic pathogens in cultured common dentex, *Dentex dentex* L. Journal of Fish Diseases **22:**299-309.
109. **FAO.** 2006-2013. Cultured aquatic species information programme. *Lates calcarifer*.
110. **Stride, M. C., A. Polkinghorne, T. L. Miller, and B. F. Nowak.** 2013. Molecular characterisation of '*Candidatus* Similichlamydia latridicola gen. nov., sp. nov.' (*Chlamydiales*: *Parilichlamydiaceae*), a novel *Chlamydia*-like epitheliocystis agent in striped trumpeter, *Latris lineata* (Forster). Applied and Environmental Microbiology **79:**4914-4920.
111. **Polinski, M., A. R. Bridle, and B. F. Nowak.** 2013. Temperature-induced transcription of inflammatory mediators and the influence of Hsp70 following LPS stimulation of southern bluefin tuna peripheral blood leukocytes and kidney homogenates. Fish & Shellfish Immunology **34:**1147-1157.
112. **Miller, M. A., W. Pfeiffer, and T. Schwartz.** 2010. Presented at the Proceedings of the Gateway Computing Environments Workshop (GCE), 14 Nov. 2010, New Orleans, LA, 14 Nov. 2010.
113. **Mitchell, S. O., T. Steinum, E. R. Toenshoff, A. Kvellestad, K. Falk, M. Horn, and D. J. Colquhoun.** 2013. '*Candidatus* Branchiomonas cysticola' is a common agent of epitheliocystis in seawater-farmed Atlantic salmon *Salmo salar* in Norway and Ireland. Diseases of Aquatic Organisms **103:**35-43.
114. **Toenshoff, E. R., A. Kvellestad, S. O. Mitchell, T. Steinum, K. Falk, D. J. Colquhoun, and M. Horn.** 2012. A novel betaproteobacterial agent of gill epitheliocystis in seawater farmed Atlantic salmon (*Salmo salar*). PLoS One **7:**e32696.

115. **Stephens, R. S., G. S. Myers, M. Eppinger, and P. M. Bavoil.** 2009. Divergence without difference: phylogenetics and taxonomy of *Chlamydia* resolved. FEMS Immunology & Medical Microbiology **55:**115-119.

116. **Greub, G.** 2010. Subcommittee on the Taxonomy of the *Chlamydiae*: Minutes of the inaugural closed meeting, 21 March 2009, Little Rock, AR, USA. International Journal of Systematic & Evolutionary Microbiology **60:**2691-2693.

117. **Camus, A., E. Soto, A. Berliner, T. Clauss, and S. Sanchez.** 2013. Epitheliocystis hyperinfection in captive spotted eagle rays *Aetobatus narinari* associated with a novel *Chlamydiales* 16S rDNA signature sequences. Diseases of Aquatic Organisms **104:**13-21.

118. **Agamy, E.** 2013. Sub chronic exposure to crude oil, dispersed oil and dispersant induces histopathological alterations in the gills of the juvenile rabbit fish (*Siganus canaliculatus*). Ecotoxicology and Environmental Safety **92:**180-190.

119. **Stride, M. C., and B. F. Nowak.** 2014. Epitheliocystis in three wild fish species in Tasmanian waters. Journal of Fish Diseases **37:**157-162.

120. **Stride, M. C., A. Polkinghorne, M. D. Powell, and B. F. Nowak.** 2013. '*Candidatus* Similichlamydia laticola', a novel *Chlamydia*-like agent of epitheliocystis in seven consecutive cohorts of farmed Australian barramundi, *Lates calcarifer* (Bloch). PLOS ONE **8** e82889.

121. **von Bomhard, W., A. Polkinghorne, Zen Huat Lu, L. Vaughan, A. Vögtlin, D. R. Zimmermann, B. Spiess, and A. Pospischil.** 2003. Detection of novel *Chlamydiae* in cats with ocular disease. American Journal of Veterinary Research **64:**1421-1428.

122. **Work, T. M., R. A. Rameyer, G. Takata, and M. L. Kent.** 2003. Protozoal and epitheliocystis-like infections in the introduced bluestripe snapper *Lutjanus kasmira* in Hawaii. Diseases of Aquatic Organisms **57:**59-66.

123. **Watanabe, K., M. Karlsen, M. Devold, E. Isdal, A. Litlaboe, and A. Nylund.** 2006. Virus-like particles associated with heart and skeletal muscle inflammation (HSMI). Diseases of Aquatic Organisms **70:**183-192.

124. **Speare, D. J., and H. W. Ferguson.** 2006. Chapter 2: Gills and pseudobranchs, p. 24-64. *In* H. W. Ferguson (ed.), Systemic Pathology of Fish: A text and atlas of normal tissues in teleosts and their responses in disease., 2nd ed. Scotian Press, London.

125. **Moyner, K., K. H. Roed, S. Sevatdal, and M. Heum.** 1993. Changes in nonspecific immune parameters in Atlantic salmon, *Salmo salar* L, induced by *Aeromonas salmonicida* infection. Fish & Shellfish Immunology **3:**253–265.

126. **Gollas-Galvan, T., L. A. Avila-Villa, M. Martinez-Porchas, and J. Hernandez-Lopez.** 2013. Rickettsia-like organisms from cultured aquatic organisms, with emphasis on necrotising hepatopancreatitis bacterium affecting penaeid shrimp: an overview on an emergent concern. Reviews in Aquaculture **5:**1-14.

127. **Corsaro, D., and D. Venditti.** 2004. Emerging Chlamydial infections. Critical Reviews in Microbiology **30:**75-106.

128. **Horn, M., and M. Wagner.** 2004. Bacterial endosymbionts of free-living amoebae. Journal of Eukayotic Microbiology **51:**509-514.

129. **Horn, M.** 2008. *Chlamydiae* as symbionts in eukaryotes. Annual Review of Microbiology **62:**113-131.

130. **Rigos, G., and P. Katharios.** 2010. Pathological obstables of newly-introduced fish species in Mediterranean mariculture: a review. Reviews in Fish Biology and Fisheries **20:**47-70.

131. **Tricklebank, K. A.** 2001. Histological alterations in fish from Sydney reefs: possible biomarkers for environmental effects? Biomarkers **6:**26-32.

132. **Kebbi-Beghdadi, K., C. Batista, and G. Greub.** 2011. Permissivity of fish cell lines to three *Chlamydia*-related bacterial: *Waddlia chondrophila*, *Estrella lausannensis* and *Parachlamydia acanthamoebae*. FEMS Immunology & Medical Microbiology **63:**339-345.

133. **Molnar, K., and G. Boros.** 1981. A Light and Electron Microscopic Study of the Agent of Carp Mucophilosis. Journal of Fish Diseases **4:**325-334.

134. **Paperna, I., and A. P. Alves de Matos.** 1984. The developmental cycle of epitheliocystis in carp, *Cyprinus carpio* L. Journal of Fish Diseases **7:**137-147.

135. **Voronin, V. N., and N. B. Chernysheva.** 1997. Epitheliocystis infection in common carp *Cyprinus carpio* L.: histopathology and pathogenicity. Bulletin of the European Association of Fish Pathologists. Weymouth **17:**137-139.

136. **Steinum, T., A. Kvellestad, D. J. Colquhoun, M. Heum, S. Mohammad, R. N. Grontvedt, and K. Falk.** 2010. Microbial and pathological findings in farmed Atlantic salmon Salmo salar with proliferative gill inflammation. Diseases of Aquatic Organisms **91:**201-211.

137. **Paperna, I., A. Colorni, B. Ross, B. Colorni, and H. Rosenthal.** 1981. Diseases of marine fish cultured in Eilat Mariculture Project based at the Gulf of Aqaba, Red Sea.

138. **Crespo, S., C. Zarza, and F. Padros.** 2001. Epitheliocystis hyperinfection in sea bass, *Dicentrarchus labrax*: light and electron microscope observations. Journal of Fish Diseases **24:**557-560.

139. **Egusa, S.** 1987. Epitheliocystis disease. Fish Pathology **22:**165-171.

140. **Ototake, M., and T. Matsusato.** 1987. Epitheliocystis found in juvenile red sea bream imported from Hong Kong. Bulletin of National Research Institute of Aquaculture **11:**51-59.

141. **Cesciha, G., E. Makovec, A. Efisio, G. Bovo, G. Ceschia, G. Giorgetti, and L. Paggi.** 1995. Epitheliocystis in sea bream (*Sparus aurata*) reared in Italy.

142. **Cruz e Silva, M. P., M. S. Freitas, and M. L. Orge.** 1997. Coinfection by monogenetic trematodes of the genus MicrotyleV.Beneden & Hesse 1863, *Lamellodiscus ignoratus* Palombi, 1943, the protozoan *Trichodina* sp.Ehrenberg, 1838 and the presence of epitheliocyctic, *Vibrio algynoliticus* and *V. Vulnificus* in cultured seabream (*Sparus aurata* L.) in Portugal. Bulletin of the European Association of Fish Pathologists **17:**40-42.

143. **Padros, F., and S. Crespo.** 1995. Proliferative epitheliocystis associated with monogenean infection in juvenile seabream *Sparaus aurata* in the North East of Spain. Bulletin of the European Association of Fish Pathologists **15:**42-44.

144. **Paperna, I., A. Colorni, H. Gordin, and G. W. Kissil.** 1977. Diseaes of *Sparus aurata* in marine culture at Elat. Aquaculture **10:**195-213.

145. **Paperna, I., I. Sabnai, and M. Castel.** 1978. Ultrastructural study of epitheliocystis organisms from gill epithelium of the fish *Sparus aurata* (L.) and *Liza ramada* (Risso) and their relation to the host cell. Journal of Fish Diseases **1:**181-189.

146. **Egusa, S., T. Miyazaki, T. Shiomitsu, and S. Fujita.** 1987. Epitheliocystis-like disease of rock seabream fry. Fish Pathology **22:**33-34.

147. **Paperna, H., I. Sabnai, and A. Zachary.** 1981. Ultrastructural Studies in Piscine Epitheliocystis: Evidence for a Pleomorphic Developmental Cycle. Journal of Fish Diseases **4:**459-472.

148. **Morrison, C., and G. Shum.** 1983. Epitheliocystis in American plaice, *Hippoglossoides platessoides* (Fabricius). Journal of Fish Diseases **6:**303-308.

APPENDIX S1: EPITHELIOCYSTIS CASES IN FISHES, THEIR GEOGRAPHICAL REGION AND SPECIFIC HOST ORIGIN (MARINE / FRESHWATER AND WILD / CULTURED), SEPARATED BY TAXONOMIC FAMILY.

Family	Geographic Region	M	FW	W	C	Reference
TRIAKIDAE						
Leopard shark (*Triakis semifasciata*)	Basel, Switzerland	X		X		(28)
MYLIOBATIDAE						
Spotted eagle ray (*Aetobatus narinari*)	Florida, USA	X		X		(117)
ACIPENSERIDAE						
White sturgeon (*Acipenser transmontanus*)	Idaho, USA		X		X	(84)
CLUPEIDAE						
Pacific herring (*Clupea pallasi*)	British Columbia, Canada	X		X		(29)
Pilchard (*Sardinops sagax*)	Australia, New Zealand	X		X		(30)
CYPRINIDAE						
Carp (*Cyprinus carpio*)	Israel, Hungry, Japan, Portugal, Russia		X		X	(23, 44, 85, 133-135)
Grass carp (*Ctenopharyngodon idella*)	Austria		X		X	(51)
CHARACIDAE						
Pacu (*Piaractus mesopotamicus*)	Brazil		X		X	(107)
ICTALURIDAE						
Brown bullhead (*Ictalurus nebulosus*)	Ontario, Canada		X	X		(31)
Channel catfish (*Ictalurus punctatus*)	Oklahoma, USA		X		X	(32)
CLARIIDAE						
African catfish (*Clarias gariepinus*)	Uganda		X		X	(48)
SALMONIDAE						
Chum salmon (*Oncorhynchus keta*)	British Columbia, Canada	X		X		(29)
Coho salmon (*Oncorhynchus kisutch*)	British Columbia, Canada Chile	X		X		(29, 33)
Pink salmon (*Oncorhynchus gorbuscha*)	British Columbia, Canada	X		X		(29)
Chinook salmon (*Oncorhynchus tshawytscha*)	British Columbia, Canada	X		X		(29)
Rainbow trout (*Oncorhynchus mykiss*)	Idaho, USA	X			X	(34)
Atlantic salmon (*Salmo salar*)	Norway, Ireland Tasmania, Australia	X	X		X	(27, 33, 50, 53, 57, 72, 113, 114, 136)
Brown trout (*Salmo trutta*)	Norway, Switzerland		X	X		(50, 101)
Arctic charr (*Salvelinus alpinus*)	British Columbia, Canada West Virginia, USA	X			X	(35, 49)
Lake trout (*Salvelinus namaycush*)	Great Lakes, USA		X		X	(36)
GADIDAE						
Fourspot flounder (*Gadus macrocephalus*)	British Columbia, Canada	X		X		(29)
Atlantic cod (*Gadus morhua*)	Northwest Atlantic, USA	X		X		(46)
Pacific tomcod (*Microgadus proximus*)	British Columbia, Canada	X		X		(29)
Walleye pollock (*Theragra chalcogramma*)	British Columbia, Canada	X		X		(29)
PHYCIDAE						
Spotted hake (*Urophycis regia*)	Northwest Atlantic, USA	X		X		(46, 56)
MERLUCCIIDAE						
Pacific hake (*Merluccius productus*)	British Columbia, Canada	X		X		(29)
BATRACHOIDIDAE						
Plainfin midshipman (*Porichthys notatus*)	British Columbia, Canada	X		X		(29)
SYNGNATHIDAE						
Leafy seadragon (*Phycodurus eques*)	Esperance, Australia	X		X		(24, 37)
Greater pipefish (*Sygnathus acus*)	England	X		X		(38)
PLATYCEPHALIDAE						
Bartail flathead (*Platycephalus sp.*)	Japan	X		X		(75)
Sand flathead (*Platycephalus bassensis*)	Tasmania, Australia	X		X		(119)
Tiger flathead (*Neoplatycephalus richarsoni*)	Tasmania, Australia	X		X		(119)
SEBASTIDAE						
Rougheye rockfish (*Sebastes aleutianus*)	British Columbia, Canada	X		X		(29)
Pacific ocean perch (*Sebastes alutus*)	British Columbia, Canada	X		X		(29)
Redbanded rockfish (*Sebastes babcocki*)	British Columbia, Canada	X		X		(29)
Silvergrey rockfish (*Sebastes brevispinis*)	British Columbia, Canada	X		X		(29)
Darkblotched rockfish (*Sebastes crameri*)	British Columbia, Canada	X		X		(29)
Greenstriped rockfish (*Sebastes elongatus*)	British Columbia, Canada	X		X		(29)
Yellowtail rockfish (*Sebastes fladivus*)	British Columbia, Canada	X		X		(29)
Canary rockfish (*Sebastes pinniger*)	British Columbia, Canada	X		X		(29)
Redstripe rockfish (*Sebastes proriger*)	British Columbia, Canada	X		X		(29)
Yellowmouth rockfish (*Sebastes reedi*)	British Columbia, Canada	X		X		(29)
Yelloweye rockfish (*Sebastes ruberrimus*)	British Columbia, Canada	X		X		(29)
Sharpchin rockfish (*Sebastes zacentrus*)	British Columbia, Canada	X		X		(29)

Family	Geographic Region	M	FW	W	C	Reference
ANOPLOPOMATIDAE						
Sablefish (*Anoplopoma fimbria*)	British Columbia, Canada	X		X		(29)
HEXAGRAMMIDAE						
Lingcod (*Ophiodon elongatus*)	British Columbia, Canada	X		X		(29)
LATIDAE						
Barramundi (*Lates calcarifer*)	Cardwell, Australia		X		X	(24, 71)
MORONIDAE						
Sea bass (*Dicentrarchus labrax*)	Mediterranean, France	X			X	(39, 137, 138)
White perch (*Morone americanus*)	Connecticut, USA		X	X		(40)
Striped bass (*Morone saxatilis*)	Connecticut, USA		X	X		(40, 56)
TERAPONTIDAE						
Silver perch (*Bidyanus bidyanus*)	Eastern Australia		X		X	(24, 41)
CENTRARCHIDAE						
Bluegill (*Lepomis macrochirus*)	Pennsylvania, USA		X		X	(55)
Largemouth bass (*Micropterus salmoides*)	Arkansas, USA		X		X	(100)
DINOLESTIDAE						
Long-finned pike (*Dinolestes lewini*)	Tasmania, Australia	X		X		(42)
LUTJANIDAE						
Bluestripe snapper (*Lutjanus kasmira*)	Hawaii, USA	X		X		(79, 122)
SPARIDAE						
Black sea bream (*Acanthopagrus schlegeli*)	Japan	X			X	(139)
Dentex (*Dentex dentex*)	Spain	X			X	(108)
Sea bream (*Diplodus puntazzo*)	Greece	X			X	(26)
Red Sea bream (*Pagrus major*)	Hong Kong Korea	X			X	(44, 54, 140)
Red/Black Sea bream hybrid (*Pagrus major x Acanthopagrus schlegeli*)	Korea	X			X	(54)
Sea bream (*Sparus aurata*)	Portugal, Spain, Israel, Mediterranean	X			X	(39, 47, 52, 99, 137, 141-145)
SCIAENIDAE						
Weakfish (*Cynoscion regalis*)	Northwest Atlantic, USA	X		X		(46)
MULLIDAE						
Goldband goatfish (*Upeneus mollucensis*)	Israel	X		X		(69)
OPLEGNATHIDAE						
Spotted knifejaw (*Oplegnathus punctatus*)	Japan	X		X		(146)
CARANGIDAE						
Amberjack (*Seriola dumerili*)	Spain	X		X		(5, 16)
Yellowtail kingfish (*Seriola lalandi*)	South Australia, Australia	X			X	(17, 70)
Yellowtail (*Seriola mazatlana*)	Ecuador	X			X	(17)
Japanese yellowtail (*Seriola quinqueradiata*)	Japan	X			X	B. Nowak (Unpublished)
Jack mackerel (*Trachurus declivis*)	Tasmania, Australia	X		X		(42, 119)
APLODACTYLIDAE						
Rock cale (*Crinodus lophodon*)	Sydney, Australia	X		X		(74)
CHEILODACTYLIDAE						
Red morwong (*Cheilodactylus fuscus*)	Sydney, Australia	X		X		(74)
LATRIDAE						
Striped trumpeter (*Latris lineata*)	Tasmania, Australia	X			X	(73, 110)
MUGILIDAE						
Golden grey mullet (*Liza aurata*)	Israel	X			X	(69, 137)
Thinlip grey mullet (*Liza ramada*)	Mediterranean, Israel	X		X		(52, 137, 145)
Greenback mullet (*Liza subviridis*)	Mediterranean	X			X	(69)
Striped mullet (*Mugil cephalus*)	Israel	X			X	(69, 137)
CICHLIDAE						
Blue/Nile tilapia hybrid (*Tilapia aurea x nilotica*)	Israel		X	X		(147)
Mozambique tilapia (*Tilapia mossambica*)	South Africa		X		X	(69, 147)
Nile tilapia (*Tilapia nilotica*)	Israel		X	X		(39, 69)
EMBIOTOCIDAE						
Shiner perch (*Cymatogaster aggregata*)	British Columbia, Canada	X		X		(29)
POMACENTRIDAE						
White-ear scalyfin (*Parma microlepis*)	NSW, Australia	X		X		(43)
ZOARCIDAE						
Ocean pout (*Macrozoarces americanus*)	Northwest Atlantic, USA	X		X		(46)
SIGANIDAE						
Rabbit fish (*Siganus canaliculatus*)	United Arab Emirates	X			X	(118)
STROMATEIDAE						
Butterfish (*Peprilus triacanthus*)	Northwest Coast, USA	X		X		(46)
PLEURONECTIDAE						
Arrowtooth flounder (*Astheresthes stomias*)	British Columbia, Canada	X		X		(29)
American plaice (*Hippoglossoides platessoides*)	Northwest Atlantic, USA Nova Scotia, Canada	X		X		(46, 148)
Dover sole (*Microstomus pacificus*)	British Columbia, Canada	X		X		(29)
Winter flounder (*Pleuronectes americanus*)	Northwest Atlantic, USA	X		X		(46)
Yellowtail flounder (*Pleuronectes ferrugineus*)	Northwest Atlantic, USA	X		X		(46)

Family	Geographic Region	M	FW	W	C	Reference
PARALICHTHYIDAE						
Pacific sanddab (*Citharichthys sordidus*)	British Columbia, Canada	X		X		(29)
Summer flounder (*Paralichthys dentatus*)	Northwest Atlantic, USA	X		X		(46)
Fourspot flounder (*Paralichthys oblongus*)	Northwest Atlantic, USA	X		X		(46)
SCOPHTHALMIDAE						
Windowpane (*Scophthalmus aquosus*)	Northwest Atlantic, USA	X		X		(46)
TETRAODONTIDAE						
Tiger puffer (*Takifugu rubripres*)	Japan	X			X	(44)

M – Marine; FW – Freshwater; W – Wild; C – Cultured

APPENDIX S2: DATA RELATED TO THE 35 *CHLAMYDIA* AND *CHLAMYDIA*-LIKE SEQUENCES USED FOR COMPARISON AND PHYLOGENETIC ANALYSIS IN YTK STUDY (CHAPTER 3)

Name	Isolate ID	Organisms Isolated from	Country Isolated from	bp	GenBank accession no.
Ca. Clavochlamydia salmonicola		Atlantic salmon	Ireland, Norway	1294	EF577392
Ca. Fritschea bemisiae	Falk	Whiteflies	USA	1542	AY140910
Ca. Metachlamydia	lacustris strain		Switzerland	1355	GQ221847
Ca. Piscichlamydia salmonis	C093-1 16S	Atlantic salmon	USA	1487	AY462244
Ca. Protochlamydia sp.	cvE14		France	1354	FJ976093
Ca. Rhabdochlamydia porcellionis		Terrestrial isopod	Slovenia	1366	AY223862
Ca. Rhabdochlamydia sp.	cvE88		France	1361	JF513056
Ca. Rhabdochlamydia sp.	CN808	Human (sputum)	Germany	1445	EU090709
Chlamydia muridarum	Nigg	Mouse	Maryland, USA	1552	AE002160
Chlamydia suis	S45	Pig	USA	1545	NR_029196
Chlamydia trachomatis	D-LC	Mouse	Montana, USA	1544	CP002054
Chlamydophila abortus	Ov/B577	Sheep	USA	1548	NR_036834
Chlamydophila caviae	GPIC	Guinea Pig	USA	1548	AE015925
Chlamydophila felis	Fe/145	Cat (Conjunctivitis)	USA	1548	D85702
Chlamydophila pecorum	E58	Cattle	USA	1548	NR_027576
Chlamydophila pneumoniae	CWL029		USA	1552	AE001363
Chlamydophila psittaci	Frt-Hu/Ca110	Ferret, Human	USA	1548	D85712
Chlamydiales symbiont of *Xenoturbella westbladi*		*Xenoturbella* (marine animal)	Sweden	1180	EF177461
Criblamydia sequanensis		Water samples	France	1479	DQ124300
Endosymbiont of *Acanthamoeba* sp.	UWE1	Human (cornea)	Washington, USA	1495	AF083614
Endosymbiont of *Acanthamoeba* sp.	UWC22	Soil sample	Washington, USA	1488	AF083616
Endosymbiont of *Bemisia tabaci*	biotype Jatropha		Arizona, USA	1511	AF400484
Estrella lausannensis	CRIB 30	Raw surface water	Spain	1468	EU074225
Neochlamydia hartmannellae	A1Hsp	Water conduit system	Germany	1529	NR_025037
Parachlamydia acanthamoebae	UV7		Austria	1551	FR872580
Parachlamydia sp.	OEW1	Saline lake water	Austria	1393	AM412760
Protochlamydia naegleriophila	cvE26		France	1388	FJ976102
Rhabdochlamydia crassificans	CRIB01	Cockroach	Germany	1495	AY928092
Simkania negevensis			Austria	1546	FR872582
Uncultured bacterium	EPR4059-B2-Bc66	Ocean crust	East Pacific Rise	1430	EU491566
Uncultured *Chlamydiaceae*			Norway	1204	EF153480
Uncultured soil bacterium	530-2	Soil sample	Western Amazon	1463	AY326519
Waddlia sp.	G817	Fruit bats	Malaysia	1548	AY184804
Waddlia chondrophila			Austria	1519	FR872662
Waddliaceae bacterium	cvE65		France	1390	JF706723